Smoking and High-Fat Diet:

Risk Factors Regulating Emphysema Formation

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Abstract

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Emphysema is a complex pathology characterized by the progressive enlargement of airspaces and the destruction of alveolar walls. Multiple environmental and genetic risk factors influence the initiation and progression of this disease. Cigarette smoke has been known for a long time to be the major contributor to the development of emphysema. However, a possible impact of hypercholesterolemia on the destruction of alveoli has not been investigated. The work presented in this thesis identifies the role of hypercholesterolemia in the development of emphysema. It also elucidates new molecular mechanisms leading to the proteolytic destruction of lung tissue, secondary to cigarette smoke exposure or to a high-fat diet.

The study reported in chapter two explores the effect of cigarette smoke on the pulmonary inflammation and proteolytic response leading to the development of emphysema in guinea pigs. For the first time, we demonstrated that smoke-induced expression of Cathepsin K in the lungs contributes to the degradation of the extracellular matrix, ultimately resulting in emphysema. Studies in chapters three and four show that hypercholesterolemia is an important risk factor for pulmonary emphysema. These studies discuss the role of MAP kinases, Toll-like signaling, ceramide signaling and

proteolytic enzymes in the pathogenesis of emphysema resulting from chronic smoke exposure or from an atherogenic diet. Data obtained with a rabbit model of emphysema further demonstrate that matrix metalloproteinase-1 (MMP-1) likely plays a key role in the pathological degradation of the lung extracellular matrix.

Together, the studies presented in this thesis indicate that, in addition to cigarette smoking, hypercholesterolemia may be an important risk factor in the development of human emphysema and provide novel insights into the molecular mechanisms leading to pulmonary inflammation and alveolar destruction. In addition, our work further illustrates the crucial role of proteases in the development of emphysema and offer novel therapeutic targets for the treatment of this disease.

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List of Abbreviations

A1AT, Alpha 1-antitrypsin

ABCA1, ATP-binding cassette transporter 1

ABCG1, ATP-binding cassette sub-family G member 1

ApoE, Apolipoprotein E

ATS, American Thoracic Society

BAL, Bronchoalveolar lavage

cAMP, Cyclic adenosine monophosphate

CD4, Cluster of differentiation 4

CD8, Cluster of differentiation 8

CDC, Centers for Disease Control and Prevention

COPD, Chronic obstructive pulmonary disease

CRP, C-reactive protein

CVD, Cardiovascular disease

DI, Destructive index

DMEM, Dulbecco's modified Eagle's medium

DNA, Deoxyribonucleic acid

DNAse, Deoxyribonuclease

ER, Endoplasmic reticulum

ERK, Extracellular signal-regulated kinase

Fas receptor, Death receptor

FBS, Fetal bovine serum

FEV_1, Forced expiratory volume in one second

FGF, Fibroblast growth factor

FVC, Forced volume vital capacity

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

G-CSF, Granulocyte colony-stimulating factor

GOLD, Global Initiative for Chronic Obstructive Lung Disease

HDL, High-density lipoprotein

HDL-C, High-density lipoprotein cholesterol

H&E, hematoxylin and eosin

IDL, Intermediate-density lipoprotein

IFN-γ, Interferon-γ

IGF, Insulin-like growth factor

IL-1β, Interleukin-1β

IL-2, Interleukin-2

IL-6, Interleukin-6

IL-8, Interleukin-8

IL-13, Interleukin-13

IL-1R1, Interleukin 1 receptor, type I

IRAK1, Interleukin-1 receptor-associated kinase 1

IRAK4, Interleukin-1 receptor-associated kinase 4

JNK, c-Jun N-terminal kinase

LDL, Low-density lipoprotein

LDLr, Low-density lipoprotein receptor

MAPK, Mitogen-activated protein kinase

MCP-1, Monocyte chemoattractant protein-1

MIP-1α, Murine macrophage inflammatory protein-1 alpha

MIP-1β, Murine macrophage inflammatory protein-1 beta

MIP-2, Murine macrophage inflammatory protein-2

MLI, Mean linear intercept

MMPs, Matrix metalloproteinases

MMP-1, Matrix metalloproteinase-1, interstitial collagenase

MMP-2, Matrix metalloproteinase-2, type IV collagenase, gelatinase A

MMP-8, Matrix metalloproteinase-8, neutrophil collagenase

MMP-9, Matrix metalloproteinase-9, type IV collagenase, gelatinase B

MMP-12, Matrix metalloproteinase-12, macrophage elastase

MMP-13, Matrix metalloproteinase-13, collagenase 3

mTOR, Mammalian target of rapamycin

MyD88, Myeloid differentiation primary response gene (88)

NHIS, National Health Interview Survey

NIH, National Institute of Health

NRF2, Nuclear factor (erythroid-derived 2)-like 2

NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells

nSMase, Neutral sphingomyelinase

PGN, Peptidoglycan

Phospho-ERK, Phosphorylated extracellular signal-regulated kinase

Phospho-JNK, Phosphorylated c-Jun N-terminal kinase

Poly(I:C), Polyinosine - polycytidylic acid

qRT-PCR, Quantitative real time polymerase chain reaction

RQ, Relative quantity

RTP801 or Redd1, Regulated in development and DNA damage responses

SFRP1, Secreted frizzled-related protein 1

SLPI, Secretory leukocyte protease inhibitor

SMP30, Senescence Marker Protein-30

SOD1, Superoxide dismutase 1 [Cu-Zn]

SOD3, Extracellular superoxide dismutase [Cu-Zn]

SP-A, Surfactant protein A

SP-D, Surfactant protein D

SR-BI, Scavenger receptor class B member 1

STAT3, Signal transducer and activator of transcription 3

STAT4, Signal transducer and activator of transcription 4

TLR, Toll-like receptor

TLR-4, Toll-like receptor 4

TNF-α, Tumor necrosis factor-alpha

Th1, T helper 1

TIMPs, Tissue inhibitors of metalloproteinases

TIMP-1, Tissue inhibitor of metalloproteinase-1

TRAF6, TNF receptor associated factor 6

TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF, Vascular endothelial growth factor

VEGFR, Vascular endothelial growth factor receptor

VLDL, Very-low-density lipoprotein

Wnt, Wingless-type

Glossary

Alveoli – the final branchings of the respiratory tree that act as the primary gas exchange units of the lung.

Alveolar type I cells – epithelial cells that have long cytoplasmic extensions, which spread along the alveolar walls and comprise the thin alveolar epithelium.

Alveolar type II cells – epithelial cells of cuboidal shape that are responsible for producing surfactant that reduces surface tension throughout the lung.

Antioxidants – molecules that are capable of inhibiting the oxidation of other molecules. Antioxidants remove free radical intermediates and inhibit other oxidation reactions. They neutralize free radicals by donating one of their own electrons.

Apolipoprotein E – a ligand that mediates uptake of VLDL particles and chylomicron remnants by the liver and peripheral cells. It is involved in the normal catabolism of triglyceride-rich lipoprotein constituents.

Apoptosis – process of programmed cell death. During apoptosis, cells die in a controlled and regulated fashion. Apoptosis is different from necrosis, which is an uncontrolled cell death leading to lysis of the cells.

Atherosclerosis – a condition in which deposits of fatty substances, cholesterol, and cellular waste products accumulate in the inner lining of an artery forming a plaque.

Caspases – cysteine proteases involved in the regulation and execution of apoptosis. They are predominantly localized in the cytoplasm of the cells.

Cathepsins – proteolytic enzymes that degrade proteins of the extracellular matrix in addition to their role in protein turnover.

Ceramide – a lipid molecule, which is composed of sphingosine and a fatty acid and has been shown to contribute to the pathogenesis of emphysema.

Chronic obstructive pulmonary disease – the co-occurrence of chronic bronchitis and emphysema, a pair of commonly co-existing diseases of the lungs in which the airways become narrowed.

Chylomicron remnants – metabolic products of chylomicron particles, in which triglycerides are removed by the lipoprotein lipase. These remnants carry dietary lipids in the blood and are cholesterol-rich.

Collagen – fibrous protein and the major component of connective tissue, cartilage and bone.

Collagenases – proteolytic enzymes that are capable of degrading triple-helical fibrillar collagens into distinctive 3/4 and 1/4 fragments.

Cysteine proteases – proteases that have a common catalytic mechanism that involves a cysteine sulfhydryl group. They have been found in viruses, bacteria, plants and mammals. They function as lysosomal mediators of terminal protein degradation, participate in apoptosis, immune responses, and extracellular matrix remodeling.

Destructive index analysis – a microscopic point count technique that determines the degree of lung parenchymal destruction. This analysis is performed using a transparent sheet with 50 counting points laid on the microscopic images from the stained lung sections.

Extracellular matrix – the extracellular part of the tissue that provides structural support to the cells, segregates tissues from one another, and regulates intercellular communication.

Emphysema – progressive disease of the lungs that causes shortness of breath. It is characterized by the destruction of lung tissue, which is necessary to support the physical shape and function of the lungs. It is often caused by smoking.

Free radicals – highly reactive chemicals that contain oxygen and are released when molecules split to give products with unpaired electrons. Free radicals can damage important cellular molecules such as DNA, lipids or other parts of the cell.

Gelatinases – protelytic enzymes that are capable of degrading type IV collagen and gelatin.

High density lipoprotein – a complex of lipids and proteins that functions as a transporter of cholesterol in the blood. High levels are associated with a decreased risk of atherosclerosis.

Hypercholesterolemia – the presence of high levels of cholesterol in the blood. It is associated with the risk of atherosclerosis.

Low density lipoprotein – a complex of lipids and proteins, with greater amounts of lipid than protein, that transports cholesterol in the blood. High levels are associated with an increased risk of atherosclerosis.

LDL receptor – a cell-surface receptor that mediates the endocytosis of LDL particles and recognizes the apoprotein B100, which is located in the phospholipid layer of LDL particles, and the apolipoprotein E, which is found in chylomicron and VLDL remnants.

Lymphocytes – white blood cells (leukocytes) determine the specificity of the immune response to infectious microorganisms and other foreign substances. They bind to antigens (foreign substances) through receptor molecules on their surfaces and remove

them from the body. These inflammatory cells are present in many pathological conditions.

Macrophages – white blood cells that are differentiated from monocytes in tissues and play an important role in the pulmonary inflammation. Their function is to engulf and digest cellular debris and pathogens and stimulate other immune cells to attack infectious agents.

Matrix metalloproteinases – family of proteases that are capable of degrading proteins of the extracellular matrix such as collagen and elastin.

Matrix metalloproteinase-1, also known as interstitial collagenase – protease that degrades interstitial collagens, type I, II and III.

Matrix metalloproteinase-9, also known as gelatinase B – protease that is capable of degrading gelatin and type IV collagen.

Matrix metalloproteinase-12, also known as macrophage elastase – protease that is capable of degrading elastin and secreted by macrophages.

Mean linear intercept – a measurement technique that evaluates enlargement of air spaces. The mean linear intercept represents the average size of alveoli. This analysis is performed using a transparent sheet with 10 horizontal and 11 vertical lines laid over the microscopic images from the stained lung sections. The intercepts of alveolar walls with these lines are counted.

Neutrophils – white blood cells that are recruited to the site of injury and represent a hallmark of acute inflammation. After bacterial infection or environmental exposure, neutrophils migrate towards the site of inflammation through the blood vessels and interstitial tissue. Their function is to phagocytose microorganisms and foreign particles. Neutrophils contain a nucleus divided into 2-5 lobes.

Neutrophil elastase – serine protease that is secreted by neutrophils and is capable of hydrolyzing elastin and other proteins.

Oxidants – chemical compounds that readily transfer oxygen atoms or gain electrons in a redox (reduction-oxidation) chemical reaction.

Oxidative stress – an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.

Proteases – enzymes that hydrolyze peptide bonds. They are divided into exopeptidases (enzymes that act near a terminus of the polypeptide chain) and endopeptidases (enzymes that cleave internal peptide bonds).

Pulmonary compliance – distensibility of the lung (elastic structure). It is defined as the change in volume of the lung produced by a change in pressure across the lung. It is an important measurement in respiratory physiology.

Serine proteases – proteases in which one of the amino acids in the active site of the enzyme is serine.

Sphingomyelinase – an enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphocholine and is involved in sphingolipid metabolism.

Surfactant – a phospholipid that lines the alveoli and serves to reduce surface tension at different volumes, contributing to alveolar stability.

Toll-like receptors – proteins that play an essential role in the innate immune response. Activation of Toll-like receptor signaling through recognition of pathogen-associated molecular patterns leads to the activation of genes encoding for pro-inflammatory cytokines and chemokines.

Western-type diet – atherogenic diet that is enriched in both fat and cholesterol.

Very low density lipoprotein – rich in triglycerides lipoproteins that circulate through the bloodstream transporting triglycerides to fat and muscle tissue until the VLDL remnants are modified and converted into LDL.

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Chapter 1

Literature Review

1. Anatomy of the lungs

The lungs play an important role in sustaining life by exchanging oxygen and carbon dioxide with the environment. The right lung is divided into three lobes and the left lung into two lobes. The lungs are separated into a conducting and respiratory zone. The conducting zone contains the trachea, the bronchi, the bronchioles, and the terminal bronchioles and is involved in the conduction of air into the lungs. The respiratory zone is the site of gas exchange and contains the respiratory bronchioles, the alveolar ducts, and the alveoli (Bittar, 2002).

The main functional units of the lungs are alveoli, which constitute 80 to 85% of the volume of normal lung (Bourbon, 1999) **(Figures 1 and 2)**. There are two important cell types lining the alveoli: alveolar type I cells and alveolar type II cells. Alveolar type I cells are thin cells that form the main structure of the alveolar wall and enable gas diffusion through close contact with capillaries. Type II cells have a cuboidal shape and secrete pulmonary surfactant, which lowers the surface tension, and thereby prevents the collapse of the alveoli during expiration (Bourbon, 1999). Alveolar macrophages are present within the alveolar air space and alveolar wall **(Figure 2)**. Their functions are to initiate immune responses and clear debris such as dust, dead cells or smoke particles (Sorokin et al., 1999). The capillary endothelium stretches out smoothly in one-cell-thick layers between the alveoli, and allows gas and fluid exchange both in and out of the bloodstream (Burri, 1999). In addition, endothelium filters blood clots and waste, moderates the action of chemicals, stores immune cells during infections and provides nutrients to lung cells. The alveolar wall contains a variety of other cell types and

extracellular fibers. These cells include fibroblasts, myofibroblasts, smooth muscle cells, and occasional mast cells.

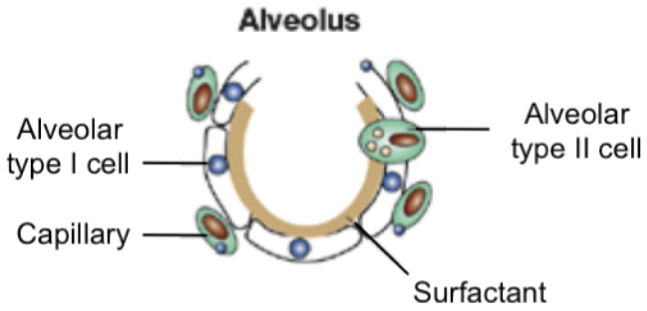

Figure 1. The primary functional unit of the lung is alveolus. Adapted from Effros RM, 2006.

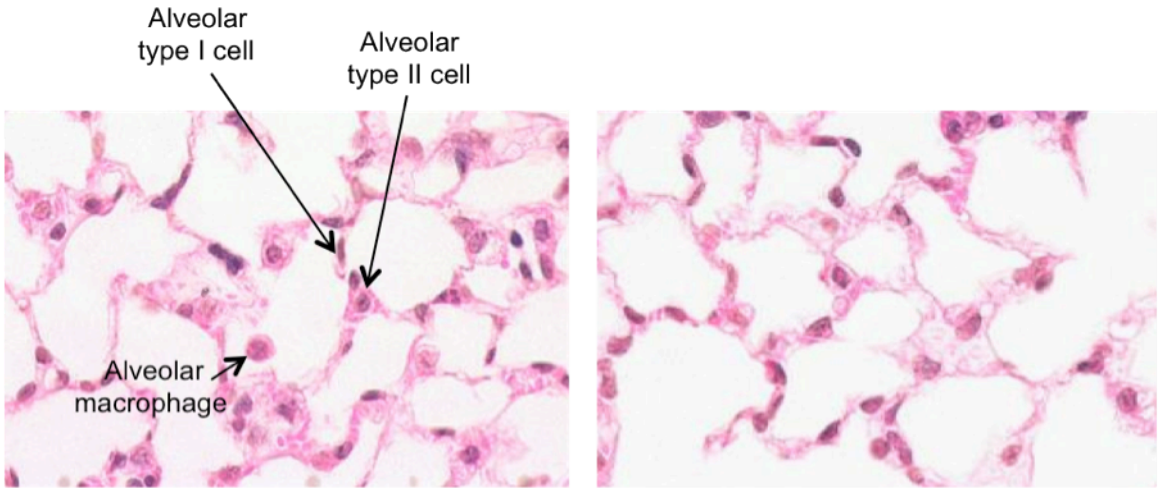

Figure 2. Histologic specimen of lung tissue stained with hematoxylin and eosin (H&E). Alveoli are lined by flattened alveolar type I cells and cuboidal alveolar type II cells. Alveolar macrophage is seen within the alveolar air space.

Fibroblasts produce Type I, II, and III collagens and elastic fibers, the major constituents of the lung extracellular matrix. Fibrillar collagens (types I, II, and III) are the most abundant collagens in the lung, but other non-fibrillar collagens are also present. The primary function of fibrillar collagens is to confer tensile strength to the distensible components of the lung, such as the large airways, blood vessels, and the alveolar interstitium (Keyzer and Post, 1999).

Type I and III collagens represent approximately 90% of all collagens in the human lung. These collagens are located throughout the alveolar interstitium, in the blood vessels, in the pleura, and around the thracheobronchial tree. Type I collagen provides tensile strength and rigidity, whereas type III forms a network of fibers and confers compliance (Keyzer and Post, 1999). Other fibrillar collagens include types II, V, and XI. Types II and XI are found in bronchial and tracheal cartilage, and type V is present in the basement membranes, interstitium, alveolar walls, and in the blood vessels (Keyzer and Post, 1999).

Type IV is the most abundant non-fibrillar collagen of the lung (Hudson et al., 1993). Contrary to fibrillar collagens, type IV collagen molecules form network structures resulting from a lateral association of their amino- and carboxy-terminal regions. These networks of type IV collagen are critical for the barrier functions of the basement membrane (Gaillard and Puchelle, 1999). This collagen is also responsible for the tensile strength of the blood-gas barrier and prevents stress failure of the pulmonary capillaries (Gaillard and Puchelle, 1999). Other non-fibrillar collagens present in the lung include type VI (in the interstitium and vessels), type VIII (in arterioles), and types IX, XII, XV, and XVIII (Laurent, 1986).

Elastin, another major component of the lung extracellular matrix, is composed of insoluble flexible cross-linked polypeptides, organized into easily extensible fibers (Fung, 1993). The elastic fibers exhibit significant structural heterogeneity and are also known to be mechanically connected to the collagen (Brown et al., 1994) via microfibrils and/or proteoglycans (Hukins, 1984; Raspanti et al., 1997; Kielty et al., 2002).

2. Chronic obstructive pulmonary disease (COPD) and emphysema

Chronic obstructive pulmonary disease (COPD) is one of the major global health problems. It represents the third leading cause of death in the United States as reported by the Centers for Disease Control and Prevention (Miniño, 2010). Currently COPD affects more than twelve million Americans (Krishnan, 2010). However, lung function tests indicate that nearly 24 million US citizens may have the disease, suggesting an underdiagnosis of COPD (Blanchette, 2011). According to the National Health Interview Survey (NHIS), the prevalence rate and the overall age-adjusted death rate for COPD is higher in the female population (Bang, Syamlal, & Mazurek, 2009). Additionally, socioeconomic status, as measured by the level of education and income, affects morbidity and mortality rates (Prescott, Lange, & Vestbo, 1999). COPD is a costly condition to treat and it was estimated that the total economic burden of this disease was $49.9 billion in 2010 (Austin, 2010). Since the prevalence of COPD and health care costs are constantly increasing, the need to better understand the mechanisms of the disease is urgent.

COPD is an umbrella term that primarily includes emphysema with enlargement of airspaces and chronic obstructive bronchitis with obstruction of small airways (Molfino, 2004). It is crucial for therapeutic and prognostic reasons to differentiate which disease state dominates in a patient. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) classifies COPD as a disease state characterized by airflow limitation that is not fully reversible and associated with an abnormal inflammatory response of the lungs to noxious particles and gases. The American Thoracic Society (ATS) classifies COPD as a disease state characterized by the presence of airflow limitation due to

chronic bronchitis or emphysema; the airflow obstruction is generally progressive and may be partially reversible ("Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society," 1995). Airflow limitation is measured by spirometry and is identified as a reduction of expiratory flow.

Emphysema is a chronic pulmonary condition that severely impacts quality of life and causes considerable morbidity in those who suffer from it (Kaplan, 2008). Emphysema is characterized by abnormal permanent enlargement of the airspaces distal to terminal bronchioles and accompanied by the destruction of alveolar walls (Vlahovic, Russell, Mercer, & Crapo, 1999). The first description of emphysema belongs to the brilliant clinician and pathologist Rene Laennec (1837). He observed that the lungs of diseased patients were hyperinflated and did not empty well. The invention of a simple spirometer helped clinicians to diagnose patients with emphysema (Hutchinson 1846) and one hundred years later the spirometer became a complete diagnostic tool for the measurement of airflow (1947). In 1944, another fundamental contributor to the knowledge of emphysema, Ronald Christie, described the major clinical features of the disease such as reduced elastic recoil and destruction of alveolar walls (Christie, 1944a, 1944b).

Presently emphysema is characterized by irreversible airflow limitation and is associated with abnormal enlargement of the airspaces distal to terminal bronchioles and accompanied by the destruction of alveolar walls (Vlahovic, Russell, Mercer, & Crapo, 1999) **(Figure 3)**. The destruction of pulmonary parenchyma is thought to result mainly from the damaging effects of proteolytic enzymes such as collagenases and elastases (D'Armiento, Dalal, Okada, Berg, & Chada, 1992; Hautamaki, Kobayashi, Senior, &

Shapiro, 1997), and free radicals generated within alveoli in response to noxious particles and gases (Rahman & MacNee, 1996). Emphysematous destruction of lung parenchyma decreases the elastic recoil force, which is necessary to expel air out of the lung (Rennard, 2006). Decreased elastic recoil of the lung causes shortness of breath, which is a common symptom among emphysema patients. Over time, the pathological changes of the lungs, such as the destruction of lung parenchyma, the loss of tissue collagen and elastin, and enlargement of alveolar airspaces may lead to the development of more advanced forms of emphysema (Suki et al., 2003). Current treatment options for COPD are limited to inhaled bronchodilators or oral corticosteroids (Niewoehner, 2010). These therapeutics can alleviate the symptoms of emphysema including breathlessness (dyspnea), wheezing, coughing and excessive mucus production, but cannot reduce the disease progression. People with the advanced form of emphysema are provided with surgical options such as lung volume reduction surgery or transplantation (Berger et al., 2010). However, these surgeries cannot guarantee a better quality of life for patients (Berger et al., 2010).

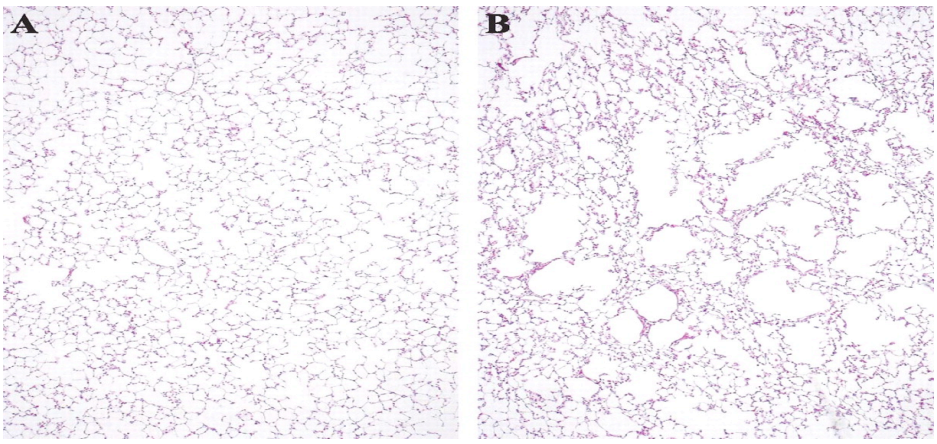

Figure 3. H&E stained lungs with normal architecture (A) and with emphysema (B). Adapted from Churg A et al., 2008.

2. Classification of COPD

COPD incorporates many complex and often overlapping respiratory conditions that develop progressively. COPD includes patients with chronic bronchitis, emphysema and a subset of patients with asthma (Mannino, 2002). Airflow obstruction as a common characteristic of COPD always should be confirmed by spirometry to avoid misdiagnosis (Holleman & Simel, 1995). The most current classification of the stage and severity of COPD is based on the level of airflow obstruction **(Table 1)**. Airflow obstruction is defined as a ratio of postbronchodilator FEV_1 (forced expiratory volume in one second) to FVC (forced volume vital capacity) of less than 0.70. However, spirometry is crucial for the diagnosis of COPD (Niewoehner, 2010). Other clinical methods evaluating the frequency of exacerbations and the intensity of breathlessness are required for the accurate interpretation of COPD (Mahler & Wells, 1988; Niewoehner et al., 2007).

Table 1. Classification of COPD. Adapted from Niewoehner DE, 2010.

Stage and Severity of COPD	Definition
Stage 1 – mild	FEV_1:FVC<0.70, FEV_1>80% of predicted value
Stage 2 – moderate	FEV_1:FVC<0.70, FEV_1 50 to 79% of predicted value
Stage 3 – severe	FEV_1:FVC<0.70, FEV_1 30 to 49% of predicted value
Stage 4 – very severe	FEV_1:FVC<0.70, FEV_1<30% of predicted value or FEV_1<50% of predicted value plus chronic respiratory failure

3. Risk factors for emphysema

Environmental or genetic risk factors may contribute to the development of emphysema. Environmental factors include tobacco smoking, occupational exposure, air pollution, socioeconomic status, diet and alcohol consumption. Genetic or acquired individual characteristics that may cause the development of emphysema include gender, genetic factors, childhood respiratory problems and familial history.

Tobacco Smoking

Tobacco smoking exposure accounts for 80-90% of COPD patients in the United States and is considered to be the primary contributing factor to the formation of emphysema (Sethi & Rochester, 2000). Tobacco smoke is comprised of about 5,000 various chemical compounds and condensed tar particles (Church & Pryor, 1985). Many of these chemicals are powerful oxidants and inducers of an inflammatory response causing lung damage. Cigarette smoke can also inhibit lung repair responses, which may lead to tissue destruction - a defining feature of emphysema (Seagrave, 2000). Results from prospective studies completed in the United Kingdom over 23 years demonstrate that smoking increases the rate of lung function decline in both males and females (Kohansal et al., 2009). The study also indicates that cessation of smoking at any age slows the decline in lung function. Of note, the presence of respiratory impaired symptoms at baseline identifies a group of smokers susceptible to the development of airflow limitation. In addition, maternal smoking has been shown to affect the respiratory function in neonates and it is likely that parental smoking confers increased risk of lower respiratory infections in children (D. G. Cook & Strachan, 1999; Hanrahan et al., 1992).

Occupational exposure

Chronic exposure to chemical substances, fumes and dusts in the work place has been shown to contribute to the development of COPD. Occupational exposure to chemical fumes or biologically inactive dust increases the prevalence of chronic airflow obstruction, mortality from COPD and increases rates of decline of FEV (Becklake, 1989; Rothenbacher et al., 1997). The health risks are elevated for miners exposed to coal dust, which accumulates within the lungs and causes emphysema (Attfield, 1985). According to the American Thoracic Society, the population risk of acquiring COPD due to occupational exposure is estimated at around 15% (Balmes et al., 2003).

Air pollution

Air pollution is believed to play a role in the development of COPD. It has been demonstrated that long-term exposure to air pollution is positively associated with the incidence of COPD (Andersen et al., 2011). Moreover, living in an urban area constitutes a considerable risk for the development of respiratory problems (Viegi et al., 1991). In addition, it has been demonstrated that people exposed to air pollution chronically or living in the polluted cities are shown to have a decline in lung function (Dockery et al., 1993).

Socioeconomic status

A low socioeconomic level is associated with the high prevalence of chronic bronchitis, emphysema and increased mortality from these diseases, which indicates that socioeconomic factors operating from early childhood affect the risk of developing COPD in adulthood independently of smoking (Prescott et al., 1999). COPD has been

found to be more prevalent among those with low income, poor education, and hazardous work or living conditions (Rogot, Sorlie, & Johnson, 1992). In addition, studies demonstrate that people with only primary and secondary education more frequently develop airflow limitations when compared with college graduates (Bakke, Hanoa, & Gulsvik, 1995).

Diet and alcohol consumption

The association between COPD and weight loss has been investigated for a long time (Donahoe & Rogers, 1990). Multiple studies have demonstrated malnutrition and reduced body weight in patients with COPD (Gray-Donald, Gibbons, Shapiro, Macklem, & Martin, 1996; Wilson, Rogers, Wright, & Anthonisen, 1989). In addition, the increased consumption of alcohol is correlated with lower levels of FEV1 and COPD exacerbation (Garshick, Segal, Worobec, Salekin, & Miller, 1989; C. C. Greene et al., 2008).

Genetic Factors

Alpha 1-antitrypsin (A1AT) deficiency is an autosomal recessive genetic disorder that leads to the abnormal production of A1AT (Gooptu, Ekeowa, & Lomas, 2009). A1AT deficiency is also known as an ER stress-related disease as it involves mutations that cause A1AT misfolding, resulting in retention of abnormal A1AT in the ER and low circulating levels of A1AT (C. M. Greene et al., 2008). Alpha 1-antitrypsin inhibits neutrophil elastase activity involved in neutrophil recruitment and destruction of pulmonary elastin. Therefore, the excessive activity of neutrophil elastase in the absence of A1AT can lead to the development of emphysema (Crystal, 1990). Smoking significantly enhances the development of emphysema not only in the homozygous but also in the heterozygous forms of the disease (Silverman et al., 1998). Other genetic

alterations such as alpha-2-macroglobulin, vitamin D binding protein and the blood serotype group genes are linked to a higher risk of acquiring emphysema (Sandford, Weir, & Pare, 1997).

Childhood and Adulthood Respiratory Problems

Numerous studies identify the association between childhood respiratory problems and reduced lung function in adulthood (Krzyzanowski, Sherrill, & Lebowitz, 1990). It has also been demonstrated that the diseases of lower respiratory tract and childhood respiratory infections during the first years of life lead to the development of impaired respiratory function and increased mortality from COPD later in life (Paoletti et al., 1989).

4. Pathogenesis of emphysema

The pathogenesis of emphysema is the subject of ongoing research because many aspects of this disease remain poorly understood. Several pathological events have been implicated in the development of emphysema, including an inflammatory proteolytic response (Grumelli et al., 2004; Shapiro, 1995), cellular apoptosis and senescence (Sato et al., 2006; Tuder, Zhen et al., 2003), oxidative stress (Rahman, Morrison, Donaldson, & MacNee, 1996), and malnutrition (D. Massaro, Massaro, Baras, Hoffman, & Clerch, 2004) **(Figure 4)**. Clinical and experimental studies using animal models, together with an association between human emphysema and deficiency in alpha1-antitrypsin indicate that proteases are major contributors to the development of emphysema. Alveolar septae of emphysematous lungs are infiltrated by inflammatory cells, including macrophages, lymphocytes, and neutrophils, which are an important source of proteases (Hogg et al., 2004). Moreover, lung epithelial and endothelial cells have also been shown to secrete proteases, contributing to the destructive airspace enlargement characteristic of emphysema (Mercer, Kolesnikova, Sonett, & D'Armiento, 2004; Taraseviciene-Stewart et al., 2005). Emphysema is also typified by a loss of alveolar capillary endothelial cells, and therefore endothelial dysfunction could be an important event contributing to emphysema (Kasahara et al., 2001). Cytokines and proteases released by inflammatory cells, apoptosis, and inhibition of the vascular endothelial growth factor (VEGF) signaling have been implicated in endothelial dysfunction in the lungs (Kanazawa, 2007). Analysis of lung tissue sections from patients with emphysema reveal elevated apoptotic epithelial, mesenchymal and endothelial cells (Imai, Mercer, Schulman, Sonett, &

D'Armiento, 2005). A disruption of the balance between apoptosis and the replenishment of structural cells in the lung could contribute to the destruction of pulmonary tissue in response to cigarette smoke, leading to emphysema.

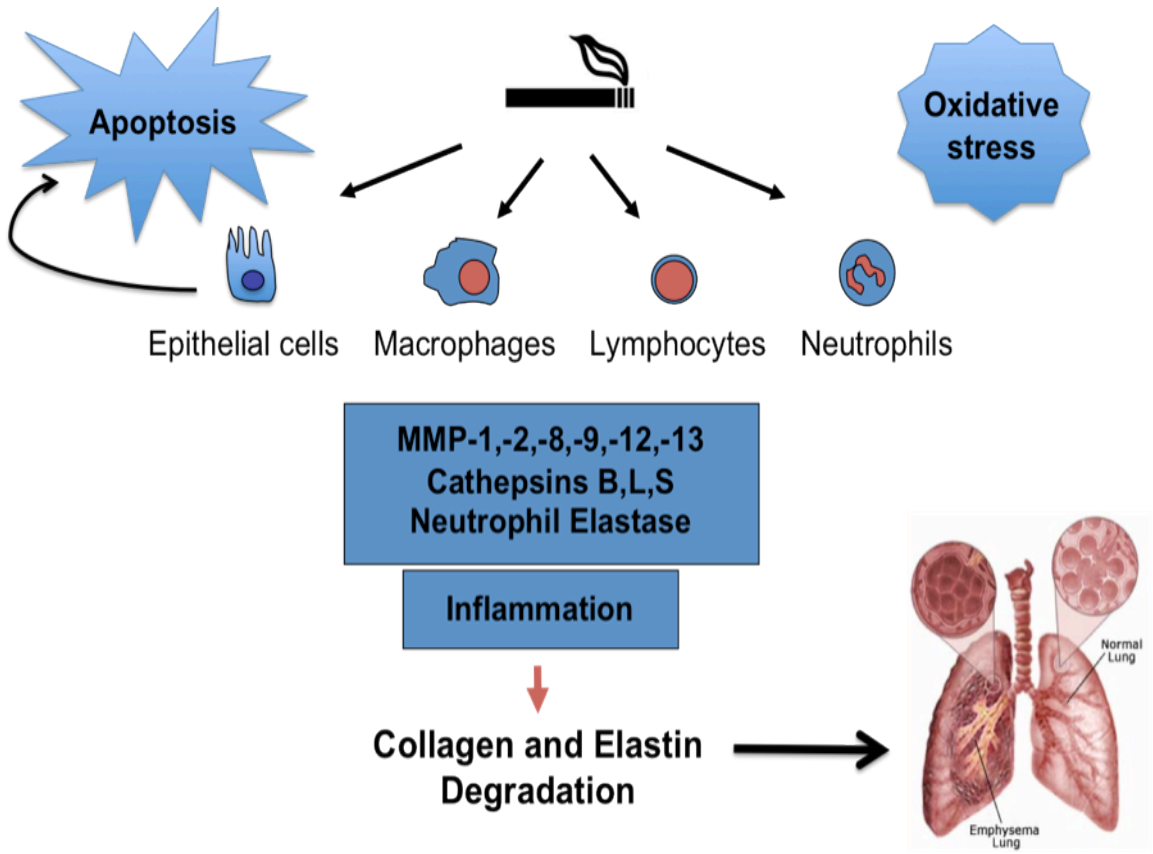

Figure 4. The Pathogenesis of COPD.

5. Contributing factors for emphysema development

5.1. Inflammation

A large number of studies suggest that the ongoing chronic inflammatory process is an important characteristic of emphysema (Hogg et al., 2004; Roth, 2008). Pathologically, pulmonary parenchyma and airways are the major sites of inflammation in emphysema. A significant body of evidence indicates that the destruction of respiratory bronchioli and alveolar walls during emphysema is the result of massive inflammation in the lung (Noble & Jiang, 2006). Potentially destructive mediators of emphysema include proteases, cytokines, and chemokines, which attract other inflammatory cells to the sites of injury. The accumulation of these inflammatory components contributes to the lung injury present in emphysema patients (Mercer et al., 2004; Saetta, Turato, Maestrelli, Mapp, & Fabbri, 2001).

Macrophages are the primary cell type regulating the inflammatory response in the lungs and are involved in tissue destruction associated with emphysema (Shapiro & Senior, 1999). A careful examination of pulmonary parenchyma of emphysema patients indicates a 25-fold increase in the number of macrophages in the tissue and alveolar space (Retamales et al., 2001). Of note, macrophages are localized mainly at the sites of inflammation or alveolar wall destruction in lungs of patients with emphysema (Finkelstein, Fraser, Ghezzo, & Cosio, 1995). Upon activation by cigarette smoke, macrophages infiltrate into alveolar septae and release a multitude of cytokines, chemokines and reactive oxygen species. These inflammatory mediators, including TNF-α, IL-8, MCP-1, orchestrate a complex inflammatory process, possibly resulting in alveolar destruction and subsequently emphysema. In addition, macrophages secrete

proteolytic enzymes that degrade major components of the lung extracellular matrix, specifically collagen and elastin, and therefore are likely to be responsible for the development of airspace enlargement (R. E. Russell et al., 2002). These alveolar macrophages release matrix metalloproteinases (MMPs) including MMP-2, MMP-9 and MMP-12, and cathepsins K, L, and S. MMPs and cathepsins are proteolytic enzymes known for their potent elastolytic and collagenolytic activity (Taggart et al., 2005; Foronjy et al., 2001; Wolters et al., 2000).

In addition to macrophages, neutrophils are strongly implicated in the pathophysiology of emphysema. Elevated numbers of activated neutrophils are found in bronchoalveolar lavage (BAL) fluid of patients with emphysema (Finkelstein et al., 1995; Lacoste et al., 1993). Clinical studies demonstrate increased concentrations of myeloperoxidase in the sputum of patients with emphysema, suggesting that neutrophils present in these lungs are activated (Keatings & Barnes, 1997; Yamamoto et al., 1997). Furthermore, the number of circulating neutrophils positively correlates with the decline in lung function, further suggesting an important role for neutrophils in human emphysema (Richards, Theron, Van der Merwe, & Anderson, 1989). After neutrophils infiltrate the airways and parenchyma, they migrate into the respiratory tract, guided by IL-8 and other neutrophil chemotactic factors. The chemotactic molecules known for their potential for neutrophil recruitment and activation include CXC chemokines, MIP-1α, MIP-1β, and MIP-2 (Tanino et al., 2002; Traves, Culpitt, Russell, Barnes, & Donnelly, 2002). Activated neutrophils have the capacity to induce lung tissue damage by releasing proteolytic enzymes and oxidants. Neutrophils secrete serine proteases (such as

neutrophil elastase) as well as MMP-8 and -9, which may cause alveolar destruction (Ilumets et al., 2008).

T lymphocytes are another cell type implicated in the inflammatory process leading to emphysema (Barnes & Cosio, 2004). The number of CD8-positive T lymphocytes is elevated in the airways and lung parenchyma of patients with emphysema (Saetta et al., 1999). The degree of airflow obstruction and the extent of the disease are also correlated with the number of these inflammatory cells, as reported by numerous studies, suggesting that these cells can potentially cause tissue injury in COPD (Finkelstein et al., 1995; Majo, Ghezzo, & Cosio, 2001). CD8-positive T cells have been implicated to cause damage to alveolar epithelial and endothelial cells in COPD patients by releasing multiple proteolytic enzymes such as perforin and granzyme A (Chrysofakis et al., 2004; Vernooy et al., 2007). CD4-positive T cells are also increased in the airways and parenchyma of COPD patients (Hogg et al., 2004; Retamales et al., 2001). Activated and oligoclonal CD4-positive T cells appear exclusively in the lung suggesting that the accumulation of these cells is a response to specific antigens present within the lung tissue (Sullivan et al., 2005). It has also been demonstrated that CD4-positive T cells express the signal transducer and activator of transcription 4 (STAT4), which induces differentiation of T cells into Th1 cells producing interferon-γ (Di Stefano et al., 2004). The number of CD4-positive T cells expressing interferon-γ positively associates with the degree of airflow obstruction in COPD patients (Di Stefano et al., 2004). The recruitment and activation of inflammatory cells, macrophages, neutrophils, CD4 and CD8 positive cells escalate as COPD worsens (Hogg et al., 2004; Retamales et al., 2001).

In addition to the inflammatory cells, epithelial and vascular endothelial cells are also important sources of inflammatory mediators and proteases involved in emphysema (Mercer et al., 2004; Taraseviciene-Stewart et al., 2005). When stimulated by cigarette smoke, these structural cells release proteases, critical for the development of emphysema, and various proinflammatory cytokines such as TNF-α and interleukins (Betsuyaku et al., 2008; Mercer et al., 2004; Rusznak et al., 2000). These cytokines amplify the recruitment of macrophages, which results in elevated expression of elastases and collagenases. Enhanced proteolytic activity within the lung causes the destruction of alveolar walls and leads to the pathological pulmonary remodeling (Suki, Lutchen, & Ingenito, 2003). Under physiological conditions, epithelial cells are involved in the survival of alveoli. They express vascular endothelial growth factor that maintains the alveolar cell survival and inhibition of its receptors may culminate in the alveolar apoptosis and emphysema-like pathology (Tuder, Zhen et al., 2003).

5.2. Protease - antiprotease imbalance

As mentioned above, proteolytic enzymes play a prominent role in the development and progression of emphysema. Numerous studies support the protease-antiprotease imbalance theory, which suggests that excessive activity of proteases or the decreased function of pulmonary antiproteases may result in the pathological destruction of the lung extracellular matrix, leading to abnormal enlargement of airspaces (Shapiro, 2003). It has been demonstrated that various proteases including cysteine proteases (cathepsins), serine proteases (neutrophil elastase) and matrix metalloproteinases degrade the connective tissue of the lung and participate in emphysema formation (D'Armiento et al., 1992; Senior et al., 1977; Wang et al., 2000).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes responsible for the degradation and remodeling of extracellular matrices during physiological and pathological events (Lemaitre & D'Armiento, 2006). Recent studies demonstrate that MMPs can also cleave non-extracellular matrix molecules such as growth factors, cytokines and their receptors (Shiomi et al., 2010). Some MMPs are membrane-anchored but most MMPs are secreted as proenzymes that require cleavage of the pro-domain for activation (Visse et al., 2003). The molecular structure of MMPs generally consists of four functional domains: signal peptide for secretion; propeptide; the catalytic domain with a conserved motif binding a zinc ion involved in proteolysis; and the C-terminal hemopexin-like domain, which mediates substrate and inhibitor interactions (Visse et al., 2003). Pro-MMPs may be activated by other active MMPs, by serine proteases such as plasmin, or by reactive oxygen species (Visse et al., 2003). Tissue inhibitors of metalloproteinases (TIMPs) play a role of antiproteases by inhibiting

MMPs in vivo. In addition, decreased activity of TIMPs can lead to excessive tissue breakdown (Shaker et al., 2008). Studies, implicating either animals or human subjects, demonstrate that decreased levels of TIMP-1 are associated with the development of pulmonary emphysema (Funada, Nishimura, & Yokoyama, 2004; Shaker et al., 2008).

The role of proteases in the development of emphysema was initially demonstrated in patients deficient in α1-antitrypsin, which is a potent inhibitor of neutrophil elastase (serine protease) (Eriksson, 1964; Morse, 1978a, 1978b). The resulting excessive activity of this elastolytic enzyme was thought to be responsible for the destruction of pulmonary elastin, causing early-onset emphysema (Senior et al., 1977). Moreover, cigarette smoke can contribute to the inhibition of α1-antitrypsin expression suggesting that even smokers without α1-antitrypsin deficiency can be affected by a protease imbalance (Carp & Janoff, 1978). Neutrophil elastase has additional functions that are important in the development of emphysema including induction of cytokine activity (IL-8) (Nakamura, Yoshimura, McElvaney, & Crystal, 1992) and impairment of macrophage ability to clear apoptotic cells (Vandivier et al., 2002). Another powerful inhibitor of serine proteases is the secretory leukocyte protease inhibitor (SLPI) (Gauthier, Fryksmark, Ohlsson, & Bieth, 1982). Its activity can be terminated either by oxidative stress or by active cathepsin B, L and S (Taggart et al., 2001). In addition, epithelial lining fluid samples from patients with emphysema demonstrate the presence of cleaved SLPI and active cathepsin L (Taggart et al., 2001). The inhibition of this cysteine protease prevents the cleavage of SLPI (Taggart et al., 2001).

The development of emphysema in mice overexpressing MMP-1 (interstitial collagenase) in the lung provides the first evidence for the importance of collagenolytic MMPs in the pathogenesis of emphysema (D'Armiento et al., 1992). These mice develop severe emphysema due to the degradation of type III collagen, one of the major components of the lung extracellular matrix (Shiomi et al., 2003), suggesting that MMP-1 may play a key role in the human disease. Bronchoalveolar fluids from patients with emphysema exhibit elevated levels of MMP-1 compared to healthy subjects (Finlay et al., 1997), and our laboratory demonstrated that MMP-1 is expressed in type II pneumocytes of human emphysematous lungs (Imai et al., 2001). Importantly, cigarette smoke extract induces the expression of MMP-1 in human lung epithelial cells in culture (Mercer et al., 2004). MMP-1 upregulation by smoke is dependent on the extracellular regulated kinase (ERK), which is activated in human emphysema (Mercer et al., 2004), and is controlled by a specific smoke responsive element in the promoter region of MMP-1 (Mercer, Wallace, Brinckerhoff, & D'Armiento, 2009). Together, these studies indicate that pulmonary MMP-1 upregulation is a critical event in the development of emphysema in smokers. Non-collagenolytic MMPs may also contribute to the disruption of the lung extracellular matrix observed in emphysema. Alveolar macrophages of patients with emphysema exhibit increased expression of MMP-9, an elastase (Finlay et al., 1997). Moreover, MMP-9 expression in alveolar macrophages of smokers is elevated compared to non-smokers, suggesting that cigarette smoke induces MMP-9 synthesis (Lim et al., 2000). It was later demonstrated in our laboratory that overexpression of MMP-9 in the macrophages of transgenic mice leads to the formation of emphysema through a loss of alveolar elastin (Foronjy et al., 2008). The significance of MMP-12 (macrophage

elastase) in the development of emphysema was first suggested in a study showing that mice deficient in this protease were protected against cigarette smoke-induced emphysema (Hautamaki et al., 1997). Furthermore, overexpression of IL-13 in MMP-12 knockout mice did not produce the significant inflammatory and emphysematous changes observed in wild-type mice (Lanone et al., 2002). These studies demonstrate that MMP-12 is important in the development of murine empnhysema. However, no increase in MMP-12 expression or activity has been found in human emphysema (Ohnishi, Takagi, Kurokawa, Satomi, & Konttinen, 1998), and therefore the exact contribution of MMP-12 in the human disease remains controversial.

Cathepsins are another class of proteases involved in the pathogenesis of emphysema and include serine, aspartate and cysteine proteases (Berdowska, 2004). Cysteine cathepsins are synthesized as preproenzymes, which are stored in lysosomes where they serve their function of protein hydrolysis (Berdowska, 2004). These enzymes contribute to distinct physiologic processes, such as antigen presentation in the immune system, collagen turnover in bone and cartilage, keratinocytes differentiation, hair follicle cycle, reproduction and apoptosis (Honey et al., 2003; Stoch, 2008; Funkelstein et al., 2008). Excessive activity of these enzymes has been detected in various pathologies, such as arthritis, cancer, atherosclerosis, and pulmonary disease (Mohamed, 2006; Lutgens, 2007). Bronchoalveolar lavage (BAL) fluids obtained from patients with emphysema contain increased concentrations of cathepsin L compared to smokers and non-smokers without emphysema (Takeyabu et al., 1998). Animal models of emphysema also indicate the likely contribution of cathepsins to emphysema development. For example, mice overexpressing IFN – γ have increased expression of cathepsins B, D, H, L, and S in their

emphysematous lungs (Wang et al., 2000). In addition, it has been shown that inhibition of cathepsin S decreases IFN – γ -induced inflammation and airspace enlargement (Zheng et al., 2005). The role of cathepsin K in collagen degradation has been demonstrated in Cathepsin K-deficient mice exhibiting decreased collagenolytic activity and development of fibrosis (Buhling et al., 2004). Together, these studies suggest a general role for cathepsins in the pathogenesis of alveolar remodeling and emphysema.

Table 2. Proteolytic enzymes (MMPs, cysteine and serine proteases), their extracellular matrix (ECM) substrates and cellular expression.

Enzymes	ECM Substrates	Cell type
Matrix Metalloproteinases (MMPs)		
Collagenases:		
Interstitial collagenase (MMP-1)	Collagen I, II, III, VII and X; gelatin; aggrecan; link protein; entactin; tenascin; perlecan	Alveolar type II cells; fibroblasts; macrophages
Neutrophil collagenase (MMP-8)	Collagen I, II, III, VII and X; gelatin; aggrecan; link protein	Neutrophils; macrophages
Collagenase-3 (MMP-13)	Collagen I, II, III, IV, IX, X and XIV; aggrecan; tenascin; osteonectin; perlecan	Macrophages; epithelial cells
Gelatinases:		
Gelatinase A (MMP-2)	Gelatin; collagen IV, V, VII, X and XI; elastin; aggrecan; link protein	Macrophages; alveolar epithelial cells; fibroblasts
Gelatinase B (MMP-9)	Gelatin; collagen III, IV and V; aggrecan; elastin; entactin; link protein; vitronectin; N-telopeptide of collagen I	Macrophages; neutrophils; mast cells; lymphocytes; NK cells; dendritic cells; bronchial epithelial cells; alveolar type II cells; fibroblasts; smooth muscle cells; endothelial cells
Stromelysin-1 (MMP-3)	Aggrecan; decorin; gelatin; collagen III, IV, IX and X; tenascin; link protein; perlecan	Macrophages; epithelial cells
Stromelysin-2 (MMP-10)	Aggrecan; collagen III, IV and V; link protein	Macrophages
Matrilysin-1 (MMP-7)	Aggrecan; gelatin; elastin; entactin; collagen IV; tenascin; decorin; link protein	Alveolar type II cells; airway epithelial cells; macrophages
Matrilysin-2 (MMP-26)	Gelatin; collagen IV; fibrinogen; vitronectin	Alveolar type II cells; epithelial cells; fibroblasts; macrophages
Stromelysin-3 (MMP-11)	Aggrecan; gelatin	Macrophages; fibroblasts
Metalloelastase (MMP-12)	Elastin; aggrecan; collagen IV; fibronectin; laminin	Macrophages; dendritic cells; lymphocytes; fibroblasts; human airway smooth muscle cells
RASI-1 (MMP-19)	Collagen IV; gelatin; tenascin; aggrecan	Smooth muscle cells; fibroblasts; macrophages; lymphocytes

Enamelysin (MMP-20)	Amelogenin; aggrecan; gelatin	Smooth muscle cells
Cysteine proteases		
Cathepsins B	Collagen II, IV, IX, X, and XI; elastin; fibronectin; laminin	Neutrophils; macrophages; fibroblasts; epithelial cells
Cathepsins D	Gelatin; collagen telopeptides; proteoglycan	Neutrophils; macrophages
Cathepsin K	Collagen I, II, III, IV, IX, and XI; elastin; gelatin; laminin; fibronectin	Macrophages; airway epithelial cells; smooth muscle cells
Cathepsins L	Elastin; collagen telopeptides; fibronectin; laminin	Neutrophils; mast cells; macrophages; epithelial cells; smooth muscle cells
Cathepsins S	Elastin; collagen telopeptides; fibronectin; laminin	Neutrophils; macrophages; airway epithelial cells; smooth muscle cells
Serine proteases		
Neutrophil Elastase	Elastin; gelatin; collagen IV; telopeptides of fibrillar collagens; proteoglycan	Neutrophils; macrophages; smooth muscle cells, endothelial cells, alveolar epithelial cells
Cathepsin G	Elastin; gelatin; collagen IV; telopeptides of fibrillar collagens; proteoglycan	Neutrophils
Proteinase 3	Elastin; gelatin; proteoglycan	Neutrophils

References: Shiomi et al., 2010; Foronjy et al., 2001; Atkinson et al., 2003; Wolters et al., 2000; Korkmaz et al., 2010; Elias et al., 2006.

5.3. Oxidative stress

Oxidative stress plays a pivotal role in the pathogenesis of pulmonary emphysema. Oxidant/antioxidant imbalance is significantly implicated in tissue destruction and apoptosis, both of which play a central role in the development of smoke-induced emphysema (Imai et al., 2005; Segura-Valdez et al., 2000). Cigarette smoke contains high concentrations of reactive oxygen species. It is estimated that each puff of cigarette smoke contains 5,000 toxic compounds, 10^{15} free radicals and potent oxidants (Church & Pryor, 1985; Pryor & Stone, 1993). These oxidant molecules damage pulmonary epithelial cells by causing direct injury to the cellular membrane and its DNA (Faux et al., 2009). Oxidative stress augments inflammation, inactivates inhibitors of proteolytic enzymes and causes apoptosis of alveolar cells ultimately leading to the formation of emphysema (Rahman et al., 2006; Buttke et al., 1994). Oxidative stress also activates the transcription factor nuclear factor-B signaling, which consequently induces proinflammatory cytokine transcription (S. R. Yang et al., 2006).

The absence of equilibrium between oxidants and antioxidants has been shown in patients with COPD (Rahman et al., 1996). A profound decrease in antioxidant capacity and elevated levels of lipid peroxidation products were demonstrated in the plasma of COPD patients and chronic smokers, but not in healthy nonsmokers (Rahman et al., 1996). Another study shows that exhaled hydrogen peroxide is elevated in the breath condensate of patients with COPD and thus provides more evidence for the presence of oxidative stress during the disease (Dekhuijzen et al., 1996). These studies highlight the role of a oxidant/antioxidant imbalance in the development and progression of COPD.

There is compelling evidence that antioxidants determine the susceptibility to smoke-induced emphysema (Rangasamy et al., 2004). Mice deficient in NRF2 (nuclear factor, erythroid-derived 2, like 2) develop profound bronchoalveolar inflammation and more extensive smoke-induced emphysema (Rangasamy et al., 2004). NRF2 is a master transcription factor, which is involved in the regulation of many detoxification and antioxidant genes (Rangasamy et al., 2004). In addition, disruption of this protein causes oxidative stress and apoptosis of alveolar septal cells, which include endothelial and type II epithelial cells (Rangasamy et al., 2004).

Research from our laboratory has identified that mice overexpressing copper-zinc superoxide dismutase (SOD1) in the lung are protected against smoke and elastase induced emphysema, suggesting an important role for this antioxidant in the disease process. SOD1, as an antioxidant, defends the lung against the damaging effects of cigarette smoke by converting superoxide into hydrogen peroxide (R. F. Foronjy et al., 2006). This study demonstrated that enhanced expression of superoxide dismutase (SOD1) leads to the attenuation of neutrophilic inflammation and oxidative damage in smoke-exposed mice. In addition, overexpression of this antioxidant counteracted proteolytic cascades involving MMP-12 and MMP-13, leading to the development of emphysema (R. F. Foronjy et al., 2006).

Additional research work provides evidence suggesting that the development of smoke-induced emphysema in mice is also dependent on the activity of the extracellular superoxide dismutase (SOD3) (Yao et al., 2010). Smoke-exposed mice deficient in SOD3 develop airspace enlargement and impaired lung function and mice overexpressing SOD3 are protected against smoke-induced inflammation and oxidative stress. These data

suggest that extracellular superoxide dismutase plays a pivotal role in protecting the lungs from oxidative stress caused by cigarette smoke (Yao et al., 2010).

In conclusion, these findings suggest that oxidative stress secondary to cigarette smoke operates as a crucial pathological mechanism linking inflammation, excessive proteolytic activity, and apoptosis in the emphysematous lung. Therefore, targeting oxidative stress with antioxidants may have beneficial outcome in the treatment of smoke-induced emphysema. However, the oxidative mechanisms involved in this pathology are very complex and need to be fully elucidated before a successful antioxidant therapy can be implemented (Rahman, 2008).

5.4. Apoptosis

Destruction of the lung extracellular matrix and alveolar walls results in airspace enlargement, which is a prominent characteristic of emphysema. In addition to excessive activity of proteases in the lung, the presence of programmed cell death or apoptosis also contributes to the development of emphysema (Imai et al., 2005; Segura-Valdez et al., 2000). There are many factors, which trigger cellular apoptosis, and among them are: loss of contact with the extracellular matrix; direct induction by inflammatory cells; and severe damages caused by various stresses (Demedts et al., 2006).

Three different signaling pathways have been involved in the regulation of apoptosis (Demedts et al., 2006). All of them result in the activation of various caspases and most importantly in the activation of caspase 3, an important protease, which excessive activity leads to epithelial apoptosis and the development of emphysematous changes (Aoshiba, Yokohori, & Nagai, 2003; Imai et al., 2005). One pathway is activated by extracellular signals and is regulated by binding of Fas, a member of tumor necrosis factor family, to death receptors positioned on the cell membrane (Demedts et al., 2006). This results in the activation of caspase-8 followed by the activation of caspase 3, which then initiates the execution of apoptosis by releasing DNAse (Hirata et al., 1998). A second pathway is the mitochondrial intrinsic pathway, which implicates the release of cytochrome C from mitochondria (Li et al., 1997). This pathway leads to the activation of caspase 9 and caspase 3. Ultimately, the endoplasmic reticulum pathway involves the activation of caspase 12 in response to stress signals (Rao et al., 2001). These molecular mechanisms play a crucial role in the maintenance of the equilibrium between cellular

apoptosis and proliferation. Disturbance of this equilibrium might contribute to the development of emphysema.

There is a body of evidence revealing the causal relationship between apoptosis and pulmonary emphysema. It has been demonstrated that the intratracheal administration of the activated caspase 3 leads to alveolar apoptosis and ultimately to the development of emphysema in mice (Aoshiba et al., 2003). Furthermore, the administration of a broad-spectrum caspase inhibitor prevents septal cell apoptosis and the development of emphysema in rats, suggesting that there is a strong link between alveolar apoptosis and pulmonary emphysema (Kasahara et al., 2000).

Another mediator of apoptosis in the pathogenesis of emphysema – besides caspase 3 – is ceramide, a lipid mediator (Petrache et al., 2005). It has been shown that ceramide triggers apoptosis of alveolar epithelial and endothelial cells, causing airspace enlargement in mice. In addition, increased levels of ceramide are found in the lungs of patients with emphysema, suggesting an important role for this highly regulated sphingolipid second messenger in the pathology of emphysema (Petrache et al., 2005).

Presence of a higher apoptosis level in human emphysematous lungs has been demonstrated in numerous studies (Imai et al., 2005; Segura-Valdez et al., 2000). In our laboratory, it has been shown that lung tissue from the patients with emphysema manifest elevated numbers of epithelial and endothelial apoptotic cells, together with an increase in caspase 3 activation (Imai et al., 2005). Another study reveals a significant increase in endothelial cell apoptosis in the lungs of COPD patients, with the less frequent appearance of apoptotic epithelial and inflammatory cells (Segura-Valdez et al., 2000).

In addition to clinical studies, animal models of emphysema also support the hypothesis that alveolar cell apoptosis contributes to the development of the disease (Kasahara et al., 2000). Blockage of VEGF receptors in a rat model of emphysema leads to apoptosis of alveolar septal cells (Kasahara et al., 2000). Mice overexpressing IFN-γ manifest emphysematous changes in their lungs and epithelial cell apoptosis. Moreover, treatment of these animals with a caspase inhibitor attenuates apoptosis and ameliorates IFN-γ-induced emphysema (Zheng et al., 2005).

In conclusion, these human and animal studies suggest that the amount of knowledge on the role of apoptosis in the pathogenesis of emphysema is growing. Yet, more studies identifying the precise molecular mechanisms aiding in the prevention and attenuation of apoptosis within the lung are needed.

5.5. Aging

There is evidence that higher prevalence of emphysema positively correlates with age (Halbert et al., 2006). Older people have been exposed to harmful gases and particles such as cigarette smoking for longer periods. As one ages, the structure and function of the lung deteriorate. The aging lung is characterized by marked structural and physiological changes (Janssens, Pache, & Nicod, 1999). Physiological pulmonary alterations are associated with a decline in elastic recoil and decreased function of respiratory muscles. The decline in elastic recoil is suggested to result from a decrease in gas exchange surface area accompanied by a loss of elastin, supporting tissue for peripheral airways (Janssens et al., 1999). Interestingly, other research suggests that morphologic changes of the aging lung include airspace enlargement but not alveolar wall destruction, which is a hallmark of emphysema (Verbeken et al., 1992a, 1992b).

Molecular mechanisms involved in the aging lung include telomere shortening, oxidative stress and DNA damage (Lee, Lu, Fahn, & Wei, 1998). A decline in cellular division capacity was attributed to the telomere shortening mechanism: telomeres that protect the ends of the chromosomes from deterioration get progressively shorter as cells divide (Holt, Shay, & Wright, 1996). It has also been demonstrated that increased levels of oxidative stress accelerate telomere shortening and, therefore, aging processes (Saretzki & Von Zglinicki, 2002). In addition, oxidative stress induces DNA damage, the accumulation of which may account for progressive deleterious pulmonary changes associated with aging (Lee et al., 1998).

Animal models with a decreased lifespan have been shown to contribute significantly to the understanding of the aging lung phenomenon. Disruption of Klotho in mice causes multiple diseases associated with aging, which include arteriosclerosis, osteoporosis and pulmonary emphysema (Kuro-o et al., 1997). Klotho protein is involved in the regulation of multiple signaling pathways, such as FGF (fibroblast growth factor) and IGF (insulin-like growth factor), mediates the activity of multiple ion channels and induces resistance to oxidative stress (Kuro-o, 2008). It has been demonstrated that the emphysematous phenotype observed in Klotho mice is likely due to the pulmonary overexpression of MMP-9 and downregulation of TIMP-1 (Funada et al., 2004). In addition, there is a possibility that the observed emphysematous changes are due to the excessive damage of oxidative stress, which results from Klotho deficiency (Kuro-o, 2008).

Senescence Marker Protein-30 (SMP30) - deficient mice develop airspace enlargement and manifest increased susceptibility to harmful stimuli. When exposed to cigarette smoke, these mice demonstrate significant parenchymal destruction and emphysematous changes (Sato et al., 2006). SMP30 is also involved in the defensive mechanism against oxidative stress and its loss is associated with an elevated expression of protein carbonyl, which is a marker of oxidative stress that increases with aging (Sato et al., 2006).

Inflammation has been documented in age-associated diseases, which include emphysema with increased recruitment of inflammatory cells and elevated circulating concentrations of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α (Ito & Barnes, 2009). In contrast, IL-2 levels are significantly decreased with aging suggesting

that immune response in elderly people is likely being impaired (Ito & Barnes, 2009). In addition, an increased number of neutrophils and the excessive production of neutrophil elastase in the lower respiratory tract of the healthy but older generation have been reported (Meyer, Rosenthal, Soergel, & Peterson, 1998).

These human and animal studies indicate a strong link between aging and the development of emphysema. Aging is a complex process influenced by various intrinsic and environmental risk factors, in particular, by oxidative stress and cigarette smoking. However, the molecular mechanisms linking aging lung and emphysema have yet to be elucidated.

6. Animal Models of Emphysema

Animal species such as guinea pigs, dogs, rats and mice have been used to study the development of emphysema (Wright, Cosio, & Churg, 2008; Chapman, 2008). There are several major experimental approaches to reproduce emphysema in vivo, which include inhalation of noxious stimuli (exposure to cigarette smoke), tracheal instillation of tissue-degrading enzymes (treatment with elastase) and gene-modifying techniques leading to emphysema-like phenotype (Wright et al., 2008).

6.1. Smoke-induced emphysema

Animal models exposed to cigarette smoke have provided valuable insights into the development of emphysema. The mode of exposure to tobacco smoke may be either active, via nose-only exposure systems, or passive via large whole-body chambers (Mauderly et al., 1989). Animal models of smoke-induced emphysema manifest dilatation of alveolar ducts and exhibit lesions similar to mild centrilobular emphysema (Cavarra et al., 2001). Measurements of air space size (morphometric analysis) and destructive index analysis should be performed on these animals to evaluate accurately the degree of lung damage. It has been shown that guinea pigs exposed to cigarette smoke for three months develop emphysema-like airspace enlargement associated with increased pulmonary compliance and total lung volumes (Wright & Churg, 1990). Interestingly, cessation of smoke exposure in the guinea pig model stabilizes but does not reverse emphysematous lung enlargement (Wright & Sun, 1994).

Rat species produce airspace enlargement with excessive elastin breakdown and pulmonary inflammation after two months of cigarette smoke exposure (Ofulue, Ko, &

Abboud, 1998). Another study involving rats examines the effects of long-term smoking on the lungs and demonstrates the development of emphysema and a loss of elastic recoil after 6 months of exposure (Huber et al., 1981).

Significant questions concerning the development of emphysema are approachable with mouse models. However, susceptibility of mice to emphysema is dependent on the genetic background (Churg, Cosio, & Wright, 2008). After 6 months of cigarette smoke exposure, C57BL/6 and A/J mice develop airspace enlargement accompanied by a significant infiltration of macrophages (Hautamaki et al., 1997). Research in our laboratory shows that CBA/J/J x C57BL/6J mice require one year of exposure to smoke in order to develop significant emphysematous changes, with a 17% increase in mean linear intercept. Another strain of mice, A/J, appears to be more susceptible to cigarette smoke and exhibits a greater loss of lung tissue, with 32% increase in mean linear intercept after only 6 months of exposure. Of note, despite this striking change in lung morphometry, compliance in the A/J mice did not change (R. F. Foronjy et al., 2005). These data suggest that the factors responsible for the development of structural emphysema may differ significantly from those that alter the physiologic characteristics of the lung.

6.2. Elastase-generated emphysema

Tracheal instillation of tissue-degrading enzymes is another experimental approach to reproduce emphysema in animals. In 1965 research work conducted by Gross et al. demonstrated that instillation of the plant protease, papain, induced emphysema in rats (Gross, Pfitzer, Tolker, Babyak, & Kaschak, 1965). This work led to

the hypothesis, for the first time, that emphysema develops as a result of excessive activity of proteases that destroy lung extracellular matrix. Intrapulmonary challenge with various proteases including porcine pancreatic elastase, papain, and human neutrophil elastase generates emphysema in animal models (Johanson & Pierce, 1973; Kaplan, Kuhn, & Pierce, 1973; Karlinsky, Goldstein, Catanese, & Snider, 1986).

The major advantage of the elastase approach is the rapid induction of emphysema. Elastase instillation can quickly produce severe emphysema with abnormalities of pulmonary function as compared to the slow smoke-induced emphysema (Wright et al., 2008). In addition, this technique can be very useful to study the effects occurring after proteolytic injury, which is necessary for the understanding of the pathology (Wright et al., 2008). However, the major disadvantage of the quick elastase-generated emphysema is that the detailed mechanism of this process is likely to be different from the mechanism of slowly developing smoke-induced emphysema in humans.

6.3. Genetically altered animal models of emphysema

Transgenic and gene targeted mice can be utilized to determine protein function in vivo and to dissect the mechanisms leading to the development of emphysema (Shapiro, 2008). Transgenic models add significantly to the field of emphysema. These animal models provide valuable knowledge about the role of inflammatory cytokines and proteases contributing to the lung destruction **(Table 3)**. Transgenic mice constitutively overexpressing human interstitial collagenase (MMP-1) manifest significant airspace enlargement, shedding light on the important relationship between collagen destruction,

collagenases and emphysema (D'Armiento et al., 1992). Mice with lung specific overexpression of TNF-α exhibit inflammation and extensive airspace enlargement accompanied by bronchiolitis. It has also been noted that the development of emphysema in these mice is due to the activation of the elastolytic MMPs, which underscores the matrix degrading functions of the MMPs (Fujita et al., 2001). Inducible overexpression of interleukin 13 (IL-13), a potent cytokine, in an adult murine lung generates profound emphysematous changes with increased pulmonary compliance and marked inflammation. Of note, the degradation of pulmonary tissue in these mice is likely due to the excessive activity of MMPs and cathepsins suggesting that IL-13 is a potent inducer of both MMPs and cathepsins during the development of emphysema (Zheng et al., 2000). Overexpression of IFN-γ in an adult murine lung leads to extensive emphysema accompanied by the infiltration of neutrophils and macrophages. The induction and activation of potent cysteine proteases and MMP-12 likely contributes to the alveolar destruction in these mice (Wang et al., 2000).

Another category of gene targeted mice does not develop emphysematous changes even if challenged with either long-term cigarette smoke exposure or tracheal elastase instillation. Mice lacking MMP-12 (macrophage elastase) not only preserve normal lung structure but also fail to generate inflammatory changes even if they are exposed to cigarette smoke for 6 months (Hautamaki et al., 1997). Neutrophil elastase-deficient mice are significantly protected (59%) against emphysema induced by cigarette smoke. In addition, these mice exhibit a decreased number of neutrophils and monocytes and low macrophage counts, suggesting that the absence of neutrophil elastase prevents not only emphysema but also inflammation in mice (Shapiro et al., 2003). These studies

emphasize the involvement of inflammatory cells and proteases in the pathogenesis of emphysema. A number of studies targeted the production and signaling of proinflammatory cytokines TNF-α and IL-1β (Churg et al., 2009; Lucey et al., 2002). These cytokines have been shown to be implicated in the development of emphysema and are consistently elevated in human smokers (Barnes, 2007; Ekberg-Jansson et al., 2001). Interestingly, after chronic exposure to cigarette smoke IL-1 receptor knockout mice are 65% protected against emphysema and mice lacking TNF-α receptors 1 and 2 are 83% protected (Churg, Zhou, Wang, Wang, & Wright, 2009). Mice deficient in both IL-1β receptor 1 and TNF-α receptors 1 and 2 are 81% protected from the development of emphysema induced by elastase instillation (Lucey, Keane, Kuang, Snider, & Goldstein, 2002). These observations further support the significant role of inflammation in emphysema formation.

Transgenic mouse models may be achieved by overexpressing certain proteins and knockout models can be generated through targeted mutagenesis. These animals can develop emphysema spontaneously with age or after being challenged with cigarette smoke. Surfactant protein D (SP-D)-deficient mice develop emphysema at 3 weeks of age. Emphysema in these mice is associated with pulmonary infiltration by cholesterol-loaded macrophages, which are known as foam cells. These lipid-loaded macrophages induce oxidative stress with consequent activation of NF-kappa-B signaling and increased MMP expression (M. Yoshida, Korfhagen, & Whitsett, 2001). Mice with lung targeted VEGF inactivation develop an emphysema-like phenotype with alveolar and bronchial cell apoptosis and loss of lung elastic recoil but without an obvious accumulation of inflammatory cells (Tang, Rossiter, Wagner, & Breen, 2004). Therefore,

these studies demonstrate an important role for SP-D and VEGF in emphysema in mice and suggest that their dysregulation could be important in the human disease.

These murine models provide a valuable insight into the cellular and molecular mechanisms that lead to the development of emphysema and explore the role of factors that mediate normal alveolar development. Together they pose the question of whether or not these mechanisms and factors work in concert or independently during the disease process.

Table 3. Rodent models of emphysema. Adapted from Taraseviciene-Stewart L., 2008.

Animal species	Intervention	Result
Mouse	Interstitial collagenase (MMP-1) overexpression	Emphysema Collagen type III degradation
Mouse	Gelatinase B (MMP-9) overexpression	Emphysema Elastin degradation
Mouse	Macrophage elastase (MMP-12) knockout	Protected against smoke-induced emphysema
Mouse	Neutrophil elastase knockout	Protected (60%) against smoke-induced emphysema
Mouse	Interleukin 1 receptor (IL-1R) knockout	Protected (65%) against smoke-induced emphysema
Mouse	Tumor necrosis factor-α receptors 1 and 2 (TNF-α R1 and R2) knockout	Protected (83%) against smoke-induced emphysema
Mouse	CuZn superoxide dismutase (CuZnSOD) overexpression	Protected against smoke-induced emphysema
Mouse	Surfactant protein D (SP-D) knockout	Emphysema Cholesterol-loaded macrophages
Mouse	Lung targeted VEGF inactivation	Emphysema Alveolar apoptosis
Mouse	Caspase-3 instillation	Alveolar wall destruction
Rat	Cigarette smoke	Emphysema and loss of elastic recoil
Rat	VEGFR blockade	Emphysema Protected by caspase inhibitor
Guinea pig	Cigarette smoke	Emphysema and vascular remodeling

7. Molecular mechanisms of emphysema

Numerous studies examine the molecular mechanisms of inflammation and protease production leading to alveolar destruction. These signaling pathways can be activated by exposure to cigarette smoke or by a variety of cytokines and growth factors. The most characterized pathways implicated in the pathogenesis of emphysema are MAPK, mTOR, Stat3, VEGF, TLR, Wnt, and NF-kB signaling (Mercer et al., 2006; Qu et al., 2009; Yoshida et al., 2010; Kasahara et al., 2001; Kneidinger et al., 2010).

Mitogen activated protein kinase (MAPK) signaling has been demonstrated not only in animal models of emphysema but also in the lungs of patients with the disease (Mercer et al., 2004). The best described MAPK signaling pathway involves extracellular regulated kinase (ERK), which is involved in many cellular processes such as cell proliferation, differentiation, motility, and death (Mercer & D'Armiento, 2006). Exposure to cigarette smoke or binding of growth factors to a receptor tyrosine kinase leads to the dimerization and autophosphorylation of the receptor, which ultimately activates MEK1/2 and ERK1/2 (Mercer & D'Armiento, 2006). There is a growing body of evidence, which suggests that activation of ERK leads to the upregulation of proteolytic enzymes implicated in the destruction of pulmonary tissue (Mercer et al., 2004).

It has been identified by our laboratory that ERK is activated in the lungs of patients with emphysema and that the induction of MMP-1, a potent protease responsible for the collagen degradation, by cigarette smoke in small airway epithelial cells is ERK-dependent (Mercer et al., 2004). Other research demonstrates that secreted frizzled-related protein 1 (SFRP1), which exhibits elevated levels in the lungs of emphysema patients, induces MMP-1 and MMP-9 expression through ERK activation (R. Foronjy et

al., 2010). These findings suggest that MAPK signaling plays an important role in the deregulation of the proteolytic balance in the lung extracellular matrix.

The mammalian target of rapamycin (mTOR) signaling also plays a role in the pathogenesis of emphysema. There is evidence demonstrating inhibition of mTOR signaling during the development of murine emphysema, which suggests that mTOR activity contributes to the maintenance of lung homeostasis (T. Yoshida et al., 2010). Overexpression in mouse lungs of RTP801, a stress-related protein triggered by adverse environmental conditions, downregulates mTOR signaling, which ultimately results in the recruitment of inflammatory cells, oxidative stress and apoptosis (T. Yoshida et al., 2010). Moreover, it has been demonstrated that administration of rapamycin, an inhibitor of mTOR signaling, leads to an increased pulmonary inflammation and induces alveolar cell apoptosis in mice (T. Yoshida et al., 2010).

Signal transducer and activator of transcription-3 (Stat3) signaling has been implicated in the pathogenesis of emphysema (Qu et al., 2009). Its been demonstrated that mice overexpressing MMP-12 develop pulmonary inflammation and spontaneous emphysema through the activation of Stat3 signaling (Qu et al., 2009). Increased MMP-12 activity leads to the inflammatory cell infiltration and increased concentrations of IL-6, which is a potent proinflammatory cytokine (Qu, Du, Wang, & Yan, 2009). IL-6 treatment augments Stat3 phosphorylation in alveolar type II epithelial cells in vivo (L. Yang et al., 2004). In addition to these observations, there is evidence that Stat3 plays a key role in MMP-1 production. These findings demonstrated that the inhibition of Stat3 signaling decreases the secretion of MMP-1 by pulmonary fibroblasts (O'Kane et al., 2010). These data suggest that activation of Stat3 signaling can significantly contribute to

the increased MMP-1 and MMP-12 activity, which might ultimately result in the development of emphysema.

Vascular endothelial growth factor (VEGF) is a survival factor for endothelial cells (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999; Senger et al., 1983). The activity of VEGF is mediated through VEGFR-1, VEGFR-2, and VEGFR-3 (Giles, 2001). VEGFR-1 is expressed on endothelial cells and mediates cell motility (Giles, 2001). The proliferative and mitogenic activities of VEGF are regulated by VEGFR-2. VEGFR-3 is believed to be involved in lymphoangiogenesis (Giles, 2001). Upon ligand binding, receptor activates a range of signaling molecules, such as phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2 (Grb2), and SH2 domain-containing phosphatases (SHP-1 and SHP-2). Activation of VEGF signaling leads to endothelial cell proliferation, vasopermeability, and angiogenesis (Giles, 2001). VEGF signaling plays a key role in the maintenance of endothelial cells and alveolar structures and under normal conditions VEGF is highly expressed in the lungs (Kasahara et al., 2001). The reduction of VEGF levels in the lungs may result in parenchymal loss, alveolar wall destruction and ultimately emphysema. In addition, it has been demonstrated that patients with emphysema have decreased expression of VEGF and endothelial cell apoptosis as compared with normal patients (Kasahara et al., 2001). Murine models also underline the importance of VEGF signaling in emphysema. Inhibition of VEGF signaling through VEGFR-2 blockade in rats leads to the destruction of pulmonary parenchyma and enlargement of alveoli (Kasahara et al., 2000). This emphysema phenotype is associated with elevated caspase-3 activity, which leads to apoptosis in the alveolar septa (Kasahara et al., 2000). Interestingly, 3-week treatment of

rats with caspase inhibitor prevents the development of emphysema, suggesting a contributory role of apoptosis in this disease (Kasahara et al., 2000). Furthermore, mice with decreased expression of pulmonary VEGF (86%) also exhibit alveolar wall destruction, increased lung compliance (loss of elastic recoil), and augmented expression of caspase-3 (Tang et al., 2004). Therefore, these observations imply that decreased VEGF signaling causes endothelial and epithelial cell apoptosis, which ultimately may result in the development of emphysema.

Toll-like receptor (TLR) signaling has a distinct role in the development of emphysema. This signaling pathway is activated in response to the products of tissue damage, viruses, and bacteria (Means et al., 2000). TLRs are expressed not only in airway and alveolar type II epithelial cells but also in alveolar macrophages and neutrophils (Armstrong et al., 2004; Fernandez, Jose, Avdiushko, Kaplan, & Cohen, 2004). This extensive expression of TLRs within the lung suggests that TLR activation is likely to contribute to the development of emphysema (Armstrong et al., 2004; Fernandez et al., 2004). In addition, ablation of IL-1R1 and MyD88, downstream regulators of TLR signaling, in mice prevents lung inflammation and pathology (Couillin et al., 2009). In contrast to the findings mentioned above, TLR-4 deficient mice develop emphysematous changes associated with oxidative stress and elastin breakdown (X. Zhang, Shan, Jiang, Cohn, & Lee, 2006). Of note, these mice do not exhibit inflammation, which is a hallmark of emphysema, suggesting that the development of the disease in this knockout model is atypical.

Wnt signaling pathway is implicated in the destruction of pulmonary tissue and defective repair mechanisms during emphysema. WNT signaling cascades can be divided

into three distinct pathways (Königshoff et al., 2010). First, the best-characterized WNT signaling pathway is the "canonical" β-catenin–dependent WNT pathway. In the absence of WNT ligands, β-catenin is attached to the scaffold proteins Axin and adenomatosis polyposis coli. It is constitutively phosphorylated via interaction with casein kinase I and glycogen synthase kinase (GSK)-3β and subsequently ubiquitinated. Second, the WNT/Ca2+ pathway signals via calmodulin kinase II and protein kinase C. Third, the WNT/JNK pathway is activated through small GTPases (Königshoff et al., 2010). It has been demonstrated that Wnt signaling is reduced in the lungs of patients with COPD and during emphysema development in mice (Kneidinger et al., 2010). Patients with COPD reveal decreased expression of the downstream regulators of Wnt signaling, such as β-catenin and Axin1 (Kneidinger et al., 2010). In addition, a recent study from our laboratory shows that an inhibitor of Wnt signaling, Secreted frizzled-related protein 1 (SFRP1), is upregulated in the lungs of patients with emphysema (R. Foronjy et al., 2010). Experimental mouse models of emphysema also confirm the involvement of this signaling pathway in this lung pathology. Elastase-treated and smoke-exposed mice exhibit decreased pulmonary expression of the main components of Wnt signaling. Moreover, activation of Wnt signaling by lithium chloride in the murine models of emphysema leads to the attenuation of airspace enlargement and improved lung physiology, which indicates a crucial role for Wnt signaling in emphysema (Kneidinger et al., 2010).

Nuclear factor-kappaB (NF-kappaB) signaling regulates a number of proinflammatory and apoptotic genes and thereby may control or contribute to the development of emphysema (Kucharczak, Simmons, Fan, & Gelinas, 2003; Tak &

Firestein, 2001). NF-kappaB proteins are normally restricted to the cytoplasm by a set of inhibitory proteins, but following receptor-mediated activation of IkappaB kinases, the inhibitors are degraded, thereby allowing nuclear import of the transcription factors that regulate multiple inflammatory cytokines and mediators (Wong et al., 2010). A significant increase in NF-kappaB expression is observed in patients with COPD compared to normal subjects suggesting an important role for this signaling in the disease (Brown, Elborn, Bradley, & Ennis, 2009). In addition, a recent work demonstrates activation of NF-kappaB signaling in the experimental mouse model of emphysema (T. Yoshida et al., 2010). These findings show that overexpression of a stress-related protein, RTP801, in mouse lungs activates NF-kappaB signaling, which may potentially lead to smoke-induced inflammation, pulmonary injury and emphysema. Moreover, RTP801-deficient mice protected against emphysematous alveolar destruction are greatly defective in NF-kappaB activation (T. Yoshida et al., 2010). These potent results emphasize the role of NF-kappaB signaling in inflammation and oxidative stress, which are characteristics of emphysema.

8. Emphysema, Diet, and Atherosclerosis

8.1. Association between atherosclerosis and emphysema (clinical studies)

The incidence of chronic obstructive pulmonary disease (COPD) in patients with cardiovascular diseases (CVD) is undervalued due to limited knowledge about the association between COPD and atherosclerosis. The development of COPD in patients with atherosclerosis considerably reduces CVD survival rates (Leavitt et al., 2006). In addition, cardiovascular morbidity and mortality rates are markedly increased in COPD patients (Sidney et al., 2005). A significant correlation between the severity of COPD and the cardiovascular morbidity and mortality has been documented (Curkendall et al., 2006). Another group of investigators report that smokers with airflow limitation exhibit severe atherosclerosis compared to smokers without airflow limitation, emphasizing the fact that reduced lung function affects the development of atherosclerosis (Iwamoto et al., 2009).

The co-morbidity of atherosclerosis and COPD is likely associated with common risk factors, such as tobacco smoking. Smoking is an established risk factor for both atherosclerosis and COPD (Barnoya & Glantz, 2005; Viegi et al., 2007). Recent research work suggests that the mechanism linking atherosclerosis and COPD can be explained by persistent systemic inflammation originating from a local infiltration of immune cells either to the vessel wall or to the airway (Sin & Man, 2003). Increased levels of C-reactive protein (CRP) are a marker for systemic inflammation in the patients with COPD and atherosclerosis. The levels of CRP are positively correlated with cardiovascular risk and poor prognosis in patients with COPD (Man et al., 2006; Ridker, Hennekens, Buring,

& Rifai, 2000). All these observations shed light on the existing link between COPD and atherosclerosis.

8.2. Animal models of atherosclerosis

There are multiple risk factors associated with the development of atherosclerosis, however, a common risk factor contributing to the complex and accelerated vascular disease in man and animals is elevated plasma cholesterol levels or hypercholesterolemia (Libby, 2002). Even though there is no animal model that would be able to entirely reproduce human-like pathology, cholesterol feeding and genetic manipulations of animals can provide valuable insights into the disease pathology (Daugherty, 2002). It has been demonstrated that genetically altered mice and rabbits are useful tools for studying the development of atherosclerosis (Daugherty, 2002; J. C. Russell & Proctor, 2006). Interestingly, in our studies these animals proved to be useful in research related to the pathogenesis of emphysema.

8.2.1. Mouse models of atherosclerosis

Mouse models are widely used to investigate the cellular and molecular mechanisms leading to the development of atherosclerosis (Daugherty, 2002). One of the reasons for such popularity is that considerable numbers of mice can be easily generated due to their short time to sexual maturity and the litter size, which ranges from six to ten pups. In addition, they are commonly used for genetic manipulations and are easy to handle and maintain. Of note, normal mice either on a chow diet or on a high-cholesterol diet are resistant to the development of severe hypercholesterolemia and atherosclerosis unless they are genetically modified (Breslow, 1996). Under normal conditions mice exhibit increased levels of anti-atherogenic HDL and decreased levels of atherogenic LDL and VLDL. Thus, the development of hypercholesterolemic mice became a

fundamental advancement in the atherosclerosis field (Breslow, 1996). The most widely used mouse models are the Apolipoprotein E knockout (*Apoe*$^{-/-}$) and the Low Density Lipoprotein Receptor knockout (*Ldlr*$^{-/-}$) mice (Daugherty, 2002).

Apolipoprotein E (ApoE) is a protein located on the surface of cholesterol-rich plasma lipoproteins and functions as a ligand for the receptors mediating hepatic clearance of the lipoproteins from the circulation (Ishibashi, Herz, Maeda, Goldstein, & Brown, 1994; Mahley, 1988). Most ApoE is generated in the liver, however, numerous peripheral tissues are also capable of synthesizing ApoE (Driscoll & Getz, 1984; Williams, Dawson, Newman, & Rudel, 1985). The role of ApoE in cholesterol homeostasis is clearly demonstrated in *Apoe*$^{-/-}$ mice. These mice develop exaggerated atherosclerosis as a consequence of their severe hypercholesterolemia (Plump et al., 1992; S. H. Zhang, Reddick, Piedrahita, & Maeda, 1992). *Apoe*$^{-/-}$ mice have five times higher total plasma cholesterol levels compared to that of normal mice even when maintained on a chow diet (434 mg/dL versus 86 mg/dL, respectively) (S. H. Zhang et al., 1992). When fed a high-cholesterol diet (Western-type diet), *Apoe*$^{-/-}$ mice increase their plasma cholesterol levels even further (ranging from 1085 to 4402 mg/dL). Whereas normal mice fed the Western-type diet do not show significantly higher levels compared to their counterparts fed chow (from 154 to 301 mg/dL versus 86 mg/dL) (Nakashima, Plump, Raines, Breslow, & Ross, 1994). This substantial elevation in plasma cholesterol levels in *Apoe*$^{-/-}$ mice is primarily due to high levels of VLDL and, to a lesser degree, LDL-sized particles (Plump & Breslow, 1995). This abnormal lipoprotein profile results from a failure to clear lipoproteins from circulation in absence of ApoE (Plump & Breslow, 1995).

LDL receptor deficiency in humans leads to a massive elevation in circulating LDL and pronounced hypercholesterolemia (Goldstein & Brown, 1974). In contrast, *Ldlr*$^{-/-}$ mice develop only a modest hypercholesterolemia when maintained on a chow diet compared to normal control mice (100 mg/dL vs 239 mg/dL respectively) (Ishibashi et al., 1993). Of note, these mice are very sensitive to dietary modifications and when fed a high-cholesterol diet they exhibit considerably higher cholesterol levels (1000 mg/dL). It has also been demonstrated that *Ldlr*$^{-/-}$ mice show a significant increase in LDL fraction and only a small elevation of VLDL particles (Ishibashi et al., 1993; Ishibashi, Goldstein, Brown, Herz, & Burns, 1994).

HDL scavenger receptor Class B Type 1 (SR-BI) regulates cellular uptake of cholesterol and other lipids from HDL particles and promotes efflux of unesterified cholesterol from cells to HDL (S. Zhang et al., 2005). Mice that are deficient in SR-BI are particularly sensitive to a high-cholesterol diet and rapidly develop high cholesterol levels and striking atherosclerosis (S. Zhang et al., 2005). Mice that are deficient in both SR-BI and ApoE and maintained on a low fat chow diet exhibit marked hypercholesterolemia (average of 1000 mg/dL) and extensive atherosclerosis suggesting the importance of these proteins in the pathogenesis of atherosclerosis (Braun et al., 2003).

8.2.2. Rabbit models of atherosclerosis

Rabbit is a valuable model to study the pathology of atherosclerosis. The rabbit rapidly develops severe hypercholesterolemia, which results in premature atherosclerosis in response to high-cholesterol feeding (Bocan et al., 1993). The typical plasma cholesterol levels in New Zealand White rabbits (NZW) are naturally low, but get significantly higher when rabbits fed the cholesterol-enriched diets (78 vs 515 mg/dl). (Daley, Herderick, Cornhill, & Rogers, 1994)

Administration of high-cholesterol diet results in a marked elevation in the production of highly atherogenic VLDL particles by the liver and intestine (MacKinnon, Savage, Gibson, & Barter, 1985; Thompson & Zilversmit, 1983). Subsequent clearance of the VLDL by the liver is decreased due to a downregulation of lipoprotein receptors (Kovanen, Brown, Basu, Bilheimer, & Goldstein, 1981). In addition, it has been demonstrated that cholesterol-fed rabbits exhibit accumulation of both VLDL and chylomicron remnants due to the low activity of LDL receptor (Hussain, Innerarity, Brecht, & Mahley, 1995).

When maintained on a high-cholesterol diet, rabbits develop hypercholesterolemia and extensive atherosclerosis (Bocan et al., 1993). It has been demonstrated that the administration of a normal chow for 9 months to the rabbits is not capable of reversing diet-induced atherosclerosis (Adams & Morgan, 1977). However, another group of researchers show that injection of HDL-C and VHDL-C can attenuate the pathology highlighting the role for HDL particles in the prevention and treatment of atherosclerosis (Badimon, Badimon, & Fuster, 1990).

There are several strains of hyperlipidemic rabbits that provide valuable insight into human disease pathology. The Watanabe heritable hyperlipidemic (WHHL) rabbit is an excellent model for understanding human familial hypercholesterolemia (Goldstein, Kita, & Brown, 1983; Watanabe, 1980). A hereditary defect in the LDL receptor causes pathological accumulation of plasma LDLs and increased cholesterol levels (400 mg/dL), which results in development of atherosclerosis (Goldstein et al., 1983). The St. Thomas Hospital strain of hyperlipidemic rabbits has increased levels of VLDL, IDL, and LDL due to overproduction of these lipoproteins, and therefore this rabbit is a good model for familial combined hyperlipidemia (Nordestgaard, Tybjaerg-Hansen, & Lewis, 1992). There are also rabbits resistant to cholesterol supplementation that do not rapidly develop atherosclerosis. When they are fed a high-cholesterol diet, the levels of plasma cholesterol, VLDL, and LDL remain unaltered (Overturf et al., 1989).

8.3. Hunger disease and emphysema

Many studies have suggested a link between malnutrition, starvation and emphysema. Malnourished patients with anorexia nervosa have been shown to possess reduced diffusing capacity and enlarged airspaces (Coxson et al., 2004). Irreversible dilation of the airways has also been noted in a patient with severe anorexia nervosa suggesting that there is a possible effect of food deprivation on the development of pulmonary pathology (V. J. Cook, Coxson, Mason, & Bai, 2001).

Numerous studies involving animals suggest that caloric restriction may result in the loss of alveoli and lung cells and that refeeding restores normal pulmonary structure (D. Massaro et al., 2004; G. D. Massaro, Radaeva, Clerch, & Massaro, 2002). Researchers believe that alveolar loss caused by starvation occurs in a regulated manner. Calorie restriction in mice aggravates the pulmonary condition up to three days and then the effect of starvation on the lung disappears even if the restriction is prolonged (D. Massaro et al., 2004). In addition, refeeding of mice with calorie-restricted intake causes spontaneous regeneration of alveoli and restoration of pulmonary tissue (G. D. Massaro et al., 2002). In contrast, animal models of smoke-induced and elastase-generated emphysema manifest the irreversible destruction of alveoli and reduced elastic recoil even after the action of the initiators of the disease is discontinued.

Other animal models of starvation have been developed to confirm the effect of caloric restriction on emphysema (Sahebjami et al., 1981; Karlinsky et al., 1986). Food restriction causes emphysematous changes in rats and hamsters (Sahebjami et al., 1981; Karlinsky et al., 1986). Hamsters produce airspace enlargement accompanied by the decrease in alveolar surface area in response to caloric restriction; however, they do not

manifest diminished elastic recoil implying that there is a difference between the physiology of smoke-induced and nutritional emphysema (Karlinsky, Goldstein, Ojserkis, & Snider, 1986). As noted above, food-restricted rats also develop emphysema. Interestingly, the severity of the disease declines in food-restricted rats depleted of protein as well (Kerr et al., 1985). These studies highlight the role of starvation in emphysema and provide valuable knowledge helping us to understand the numerous mechanisms that may contribute to the development or prevention of emphysema. These observations also demonstrate that there are many more phenotypes that need to be explained and require a detailed characterization to identify the potential treatments for this disease.

9. Significance of the present study

The goal of the present study is to better understand the molecular mechanisms leading to emphysema formation secondary to cigarette smoke and hypercholesterolemia. Cigarette smoking is a major risk factor for both chronic obstructive pulmonary disease (COPD) and cardiovascular diseases (CVD) (Barnoya & Glantz, 2005; Viegi et al., 2007). Hypercholesterolemia is a well-established risk factor for the development of atherosclerosis (Libby, 2002).

Cigarette smoke exposure induces pulmonary inflammation and causes oxidative stress, apoptosis, and an imbalance between proteolytic enzymes and their inhibitors (Sharafkhaneh, Hanania, & Kim, 2008). Excessive activity of proteases in the lungs results in the disruption of the extracellular matrix, contributing to the development of emphysema (Foronjy, 2001; Shapiro, 1999). However, the precise molecular mechanisms responsible for smoke-induced emphysema are still poorly understood. Our laboratory has demonstrated that matrix metalloproteinase-1 (MMP-1) plays a key role in this pathology. MMP-1 is expressed in human emphysema and, in transgenic mice it causes emphysema through cleavage of type III collagen (Shiomi, 2003; Imai, 2001). Importantly, cigarette smoke induces MMP-1 in human epithelial cells (Mercer, 2004). However, rodents do not express a true homologue for MMP-1, and therefore the use of other models is necessary to further study the role of this protease in smoke-induced emphysema (Henriet, Rousseau, & Eeckhout, 1992; Schorpp et al., 1995). In chapters two and four of this thesis, we examine the histologic and molecular effects of smoke exposure on the lungs of guinea pigs and rabbits, two species that express a homologue for human MMP-1 (Huebner, 1998; Vincenti, 1998).

Recent publications in peer-reviewed journals have suggested the existence of a link between COPD and CVD (Sidney et al., 2005; Leavitt et al., 2006). It has been demonstrated that cardiovascular morbidity and mortality rates are markedly increased in patients with COPD (Sidney et al., 2005). Moreover, the development of COPD in patients with atherosclerosis considerably reduces CVD survival rates (Leavitt et al., 2006). The mechanism linking atherosclerosis and emphysema could be attributed to a persistent systemic inflammation originating from a local infiltration of immune cells, either to the vessel wall or to the airway (Sin & Man, 2003).

In the present study we investigate the possible impact of hypercholesterolemia, a common feature of atherosclerosis, on the enlargement of airspaces and the destruction of alveolar walls. With 12 million patients currently diagnosed with COPD in the United States, it is essential to study the molecular mechanisms potentially linking emphysema formation and atherosclerosis (Krishnan, 2010). In chapters three and four of this thesis, we identify the role of hypercholesterolemia in pulmonary inflammation, proteolytic responses, and in the development of emphysema using *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, and rabbits.

The animal models characterized in our study will contribute to our understanding of the signaling pathways by which hypercholesterolemia and cigarette smoke induce pulmonary disease. In the future, this research may facilitate the development of new and effective therapeutics for the treatment of emphysema secondary to smoke and to hypercholesterolemia.

Chapter 2

Guinea pig model of smoke-induced emphysema

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ROLE FOR CATHEPSIN K IN EMPHYSEMA IN SMOKE-EXPOSED GUINEA PIGS

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☐ *The protease-antiprotease imbalance in the lung plays an important role in the pathogenesis of smoke-induced emphysema. The aim of this study was to characterize the proteolytic responses leading to emphysema formation in the guinea pig smoke exposure model. Guinea pigs were exposed to cigarette smoke for 1, 2, 4, 8, and 12 weeks. Age-matched guinea pigs exposed to room air served as controls. Cigarette smoke induced inflammation after 4 weeks and generated emphysematous changes in the guinea pigs after 12 weeks of smoke exposure. Increased phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinases was demonstrated post cigarette smoke exposure. A decrease in elastin and collagen and the loss of type III collagen were observed in the alveolar wall of smoke-exposed guinea pigs. Interestingly, no change was seen in the expression of collagenolytic matrix metalloproteinases. Furthermore, the authors observed a 3-fold increase in cathepsin K activity in the lungs of smoke-exposed guinea pigs. The significance of this finding was supported by human studies that demonstrate increased expression of cathepsin K in the lungs of patients with emphysema. Elevation of cathepsin K in guinea pig lungs after smoke exposure likely constitutes a critical event leading to the disruption of lung extracellular matrix in this model.*

Keywords emphysema, inflammation, proteases

Cigarette smoking is the major and most preventable cause of emphysema, a disease characterized by the abnormal enlargement of airspaces and the destruction of alveolar walls. Over 4 million people in the United States are affected by this condition [1]. To investigate the pathology of smoke-induced emphysema, the use of an adequate animal model is essential. Mice have been shown to develop emphysematous changes after exposure to cigarette smoke. The mechanism for the abnormal development of the structural

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changes in the lung extracellular matrix implicates members of serine- and cysteine-protease families and matrix metalloproteases (MMPs) [2–4]. Their dysregulated activity likely causes the pathological disruption of pulmonary collagen and elastin leading to emphysema [5]. Genetically modified mouse models overexpressing these proteases spontaneously developed emphysema [6, 7]. Transgenic mice expressing human MMP-1 developed alterations in the lung architecture, as well as emphysematous changes due to the digestion of type III collagen [8]. Induced overexpression of the inflammatory cytokine interleukin (IL)-13 in murine lungs stimulated the expression of MMPs and cathepsins, which resulted in emphysematous changes in the lung [9]. In addition, mice with a loss of MMP-12 were resistant to smoke-induced emphysema, and did not demonstrate any increase in lung inflammation and in the destruction of alveolar walls after smoke exposure [10]. These studies support the role of proteases in the development of emphysema and emphasize that multiple proteases are involved in this process.

Although mouse models have been used extensively to study emphysema, the difference in enzyme repertoire and immune response in rodents compared to higher species constitutes a major limitation in our understanding of the complex pathology of human emphysema. For instance, the murine homologue for MMP-1, a protease that is believed to contribute significantly to human emphysema, is not present in the mouse lung and its activity is not equivalent to the activity of human MMP-1 [11]. It is therefore important to characterize other animal models that may provide valuable insight into the human disease.

The contribution of the guinea pig model of smoke-induced emphysema to the understanding of human disease pathology is valuable because the development of emphysema in guinea pigs exhibits several similarities to that of the human disease [12]. The goal of this study was to characterize the guinea pig as a model of smoke-induced emphysema, in particular, to understand the effect of cigarette smoke on the lung extracellular matrix, cellular signaling pathways, and proteases expression.

MATERIALS AND METHODS

Animal Experiments

Forty male guinea pigs (Hartley strain) weighing 480 to 600 g were obtained from Charles River (Wilmington, MA). Animals were exposed to cigarette smoke (Research Cigarettes Type 2RF, University of Kentucky) for 4 hours a day, 5 days per week, for 1, 2, 4, 8, and 12 weeks at a total particulate matter (TPM) concentration of 250 mg/m^3 in a specially designed smoking chamber (Teague Enterprises, CA). For each time point, 4 animals were exposed to smoke and 4 animals were exposed to room air as

controls. The smoking machine generated 2 70-mL puffs per minute from the cigarettes and delivered them to whole body exposure chambers. The concentration of TPM whithin the chambers was at the same level (250 mg/m^3) at all times. Gravimetric analysis of filter samples taken during the exposure periods determined total particulate matter concentration in the chambers. Experiments were approved by the Institute for Animal Care and Use Committee of Columbia University.

Human Lung Tissue

Human lung samples were obtained at Columbia Presbyterian Medical Center (New York, NY) (Institutional Review Board protocol no. 9956) as previously described [4].

Histology and Immunohistochemistry

After exposure to smoke or room air, the animals were sacrificed by carbon dioxide inhalation. The trachea was cannulated with a 16-G argon catheter secured with a silk suture. The lungs were lavaged first with phosphate-buffered saline (PBS) (10 mL), to collect bronchoalveolar lavage (BAL) fluid, then with formalin for fixation. Tissues were embedded in paraffin and sectioned (6 μm). Sections were stained with hematoxylin and eosin (H&E) for histological analysis and quantification of macrophages. Serial sections were also stained with elastica–van Gieson stain for elastic fibers. For collagen quantification, the lungs of control and smoke-exposed guinea pigs were stained with Masson's trichrome. Stained areas were quantified using video-microscopy and ImagePro 4.5 software. For each animal, 4 sections equally distant from each other were used. For immunohistochemical analysis, rabbit polyclonal antibodies for type III collagen (Rockwell) [8] and cathepsin K (Biovision) were used. Quantification of collagen staining was performed using the Image-Pro 4.5 software. Morphometric analysis of the H&E (hematoxylin and eosin) stained lungs was performed as previously described [8]. We adhered to a rigorous protocol for our morphometric analysis and the lungs were pressure-perfused at 25 cm H_2O with 10% buffered formalin for 24 hours.

Western Blots

Freshly dissected guinea pig lungs (10 mg) were homogenized in 1 mL of protein lysis buffer (PBS containing Triton X-100 0.1%), and centrifuged (14000 × g for 10 minutes). Fifty micrograms of the lung lysates of each group were subjected to Western Blot analysis. Rabbit polyclonal antibodies against p-ERK (phosphorylated extracellular signal-related kinase), p-JNK

(phosphorylated c-Jun N-terminal kinase), and p-p38 (Cell Signaling) and mouse monoclonal antibody (Calbiochem) against cathepsin K were used, following the manufacturer's instructions.

Zymography

Zymography was performed to detect proteases having gelatinolytic activity, MMP-2, and MMP-9 as previously reported [13].

Cathepsin K, S, and L Activity Assays Using a Fluorescent Substrate

Cathepsin K, S, and L activity in lung extracts was assayed using specific fluorescent substrates (Assay Kits from BioVision). This kit uses specific substrate sequence Val-Val-Arg (VVR) labeled with AFS (amino-4-trifluoromethylcoumarin), which is released after proteolytic cleavage. Two microliters of 10 mM Ac-VVR-AFS substrate were added to 50 μL of each sample. Samples were run in triplicate. Specific inhibitors of cathepsins were included in the assay, as negative controls.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 2 specimens of lung tissue 0.3 cm^3 in size with the use of a RNeasy kit (Qiagen). TaqMan gene expression assays were performed to assess gene-transcript levels with the use of an ABI Prism 7900HT Sequence Detection System (Applera, Foster City, CA). Primer and probe sets were purchased from ABI and included the following: murine collagenase-A (Mcol-A; Mm00473485_m1), MMP-1 (Hs00233958_m1), MMP-8 (Mm00772335_m1), MMP-9 (Hs00234579_m1 and Mm00442991_m1), MMP-12 (Hs00159178_m1 and Mm00500554_m1), MMP-13 (Mm00439491_m1), and MMP-14 (Mm00485054_m1). β -Actin was used as the housekeeping gene. As the complete guinea pig sequence is not published we tested assays that have been developed based on the human and mouse sequences. Quantitative RT-PCR analysis was performed on lung tissue from four animals in each group.

Statistical Analysis

Data are shown as mean ± SD. Student's t test analysis was performed to determine statistical significance ($P < .05$).

RESULTS

Inflammatory Response to Cigarette Smoke in Guinea Pigs

To determine the influence of cigarette smoke on lung inflammation, macrophages and neutrophils were quantified in tissue sections of the smoke exposed guinea pigs and nonexposed controls. An increased number of macrophages was observed after 12 weeks of smoke exposure (18.64 ± 3.31 macrophages per mm^2 for the nonexposed versus 28.63 ± 4.47 macrophages per mm^2 for the smoke-exposed; $P < .001$) (Figure 1*A*). Both groups of animals had a low and similar number of neutrophils (2.39 ± 3.03 versus 1.81 ± 1.43 neutrophils per mm^2) (Figure 1*A*). This histological analysis of lung tissue sections reveals that cigarette smoke increases lung inflammation in guinea pigs, which consists primarily of macrophages and not neutrophils. At all of the time points, total BAL cell count and macrophages were increased after smoke exposure. Gelatin zymography was performed

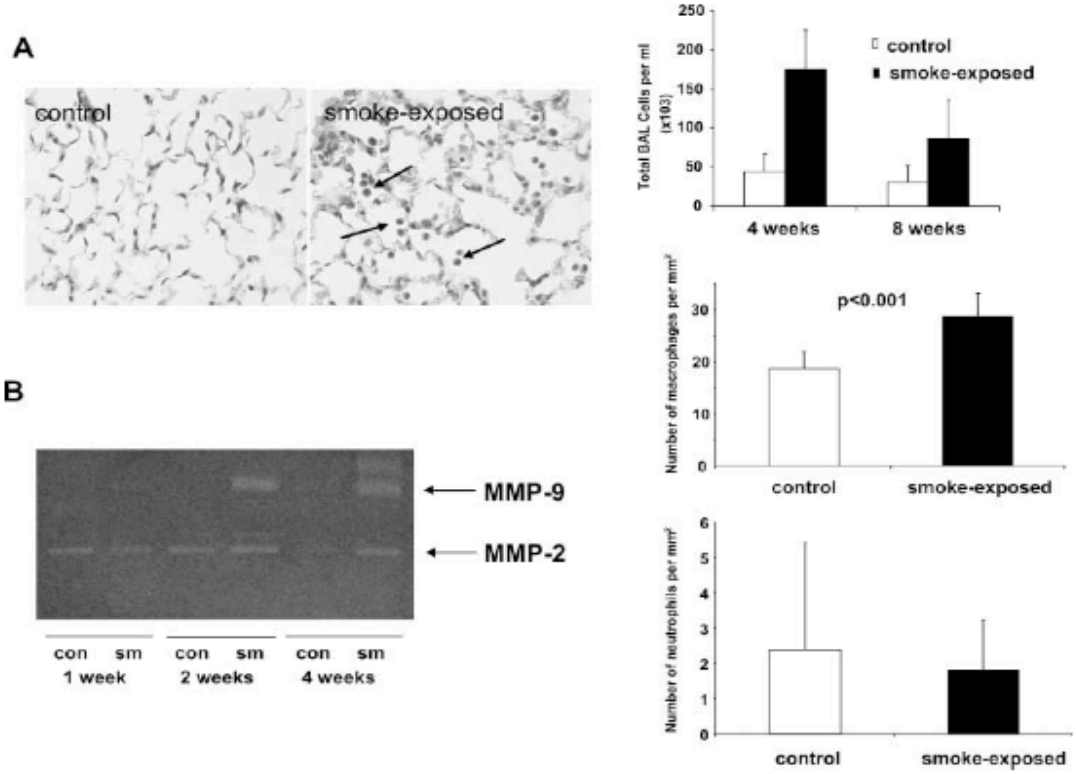

FIGURE 1 Increased macrophages and elevated MMP-9 activity in the lungs of smoke-exposed animals. (*A*) A statistically significant increase in the number of macrophages was observed in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks (18.64 ± 3.31 versus 28.63 ± 4.47 macrophages per mm^2; $P < .001$). The number of neutrophils was similar in both groups of animals (2.39 ± 3.03 versus 1.81 ± 1.43 neutrophils per mm^2). (*B*) MMP-9 activity was detected in the bronchioalveolar lavage (BAL) fluid of smoke-exposed (SM) guinea pigs at 2 and 4 weeks when compared to the control (CON).

on the BAL fluid of guinea pigs exposed to cigarette smoke for 2 and 4 weeks (Figure 1*B*). Increased activity of MMP-9 was observed, which is predictable because MMP-9 is expressed mainly in macrophages [14].

Activation of ERK and JNK in the Lungs of Guinea Pigs Exposed to Cigarette Smoke

Phosphorylation of mitogen-activated protein (MAP) kinases leads to the activation of transcription factors that participate in the regulation of inflammatory genes. Our laboratory has observed elevated phospho-ERK in both in vitro and in vivo models treated with cigarette smoke extract and in human emphysema. To determine if cigarette smoke modulates the phosphorylation of major MAP kinases in the guinea pig model, we analyzed lung lysates by Western blot (Figure 2). This analysis revealed an increase in phosphorylated-ERK and phosphorylated-JNK in the lungs of smoke-exposed guinea pigs after 4 and 12 weeks of exposure, compared with nonexposed controls (Figure 2*A* and *B*). In contrast, exposure to cigarette smoke did not affect the phosphorylation of p38 (Figure 2*B*), as is the case in the mouse model of smoke exposure [15].

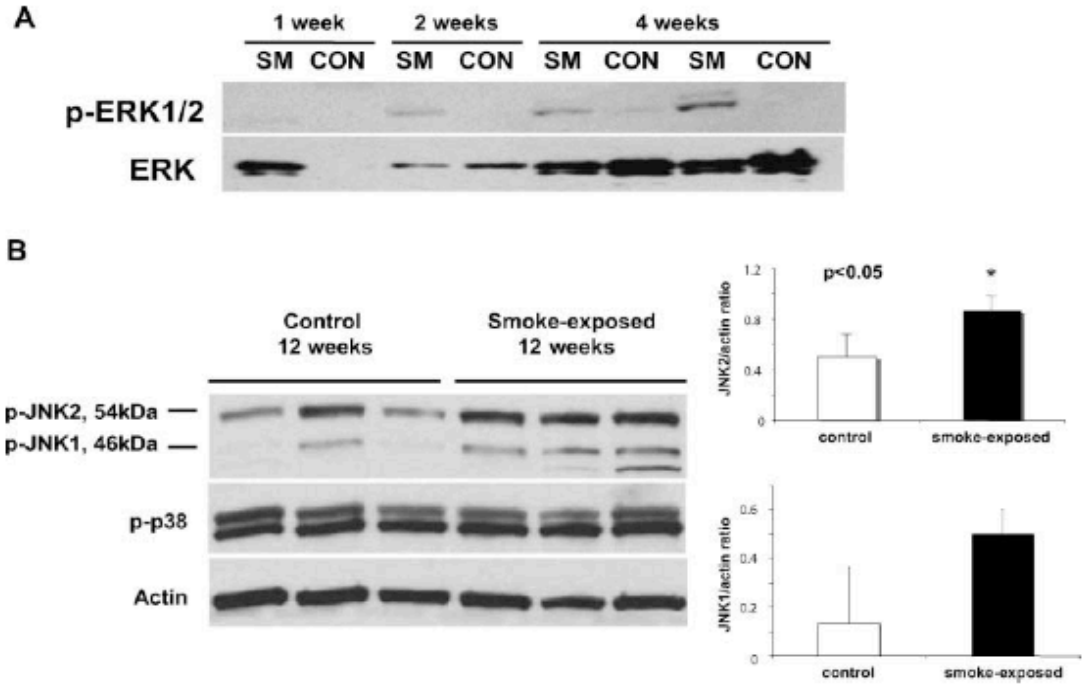

FIGURE 2 ERK phosphorylation and JNK pathways in smoke-exposed guinea pig lungs. (*A*) Increased phospho-ERK (pERK) was demonstrated after 2 weeks of smoke exposure and continues through until 4 weeks of exposure. (*B*) Increase in phospho-JNK detected after 12 weeks of smoke exposure. Densitometric analysis of the signal was performed to determine JNK/actin ratio.

TABLE 1 Morphometry Measurements of Nonexposed and Smoke-Exposed Guinea Pigs (12 Weeks of Smoke Exposure)

Guinea pigs	Surface Area/Unit Volume	Fractional Volume %
Nonexposed	79.5 ± 5	42.1 ± 8.4
Smoke-exposed	61.2 ± 1.6*	42 ± 8.6

Note. A statistically significant decrease of the alveolar surface area was detected in the smoke-exposed animals. *$P < .003$.

Development of Emphysema Post Cigarette Smoke Exposure in Guinea Pigs

The impact of cigarette smoke on the development of emphysema in guinea pigs was determined by measuring the mean linear intercept of the airspaces in lungs of smoke-exposed ($n = 4$) and control animals ($n = 4$) (Figure 3*A*). Our morphometric analysis showed that guinea pigs exposed to cigarette smoke for 12 weeks developed statistically significant airspace enlargement compared with the controls (32.5 μm versus 25 μm for the controls; $P < .001$) (Figure 3*A*). A destructive index analysis showed a similar pattern when compared to the morphometric assessment of the guinea pig lungs (67% versus 51% for the controls; $P < .001$) (Figure 3*A*). In addition, a marked decrease in the alveolar surface area was observed in the lungs of smoke-exposed animals (Table 1). Animals exposed to cigarette smoke for 8 weeks did not exhibit emphysematous changes in their lungs (data not shown). No evidence of apoptosis was detected by TUNEL (deoxynucleotidyltransferase-mediated dUTP nick end labeling) assay and there was no increase in caspase activity (data not shown).

Decrease in Pulmonary Collagen and Elastin in Smoke-Exposed Guinea Pigs

To assess the influence of cigarette smoke on the lung extracellular matrix, histological staining for collagen and elastin was performed. We observed a significant decrease in signal intensity in the alveolar walls of smoke-exposed animals, where fibers of collagen and elastin were thinner (Figure 3*B* and *C*). Quantification of the stained collagen using videomicroscopy demonstrated a very significant decrease of collagen content due to smoke exposure (12.7% ± 6% of total tissue area for the smoke-exposed animals, compared to 37.6% ± 18% for the controls; Figure 3*B*). Proteolytic degradation of type III collagen has been seen in mouse models of emphysema [8]. In the guinea pig smoke exposure model, immunohistochemistry demonstrated a marked decrease in type III collagen on lung sections of smoke-exposed guinea pigs (Figure 3*C*). These data provide evidence that cigarette smoke affects the integrity of the lung extracellular

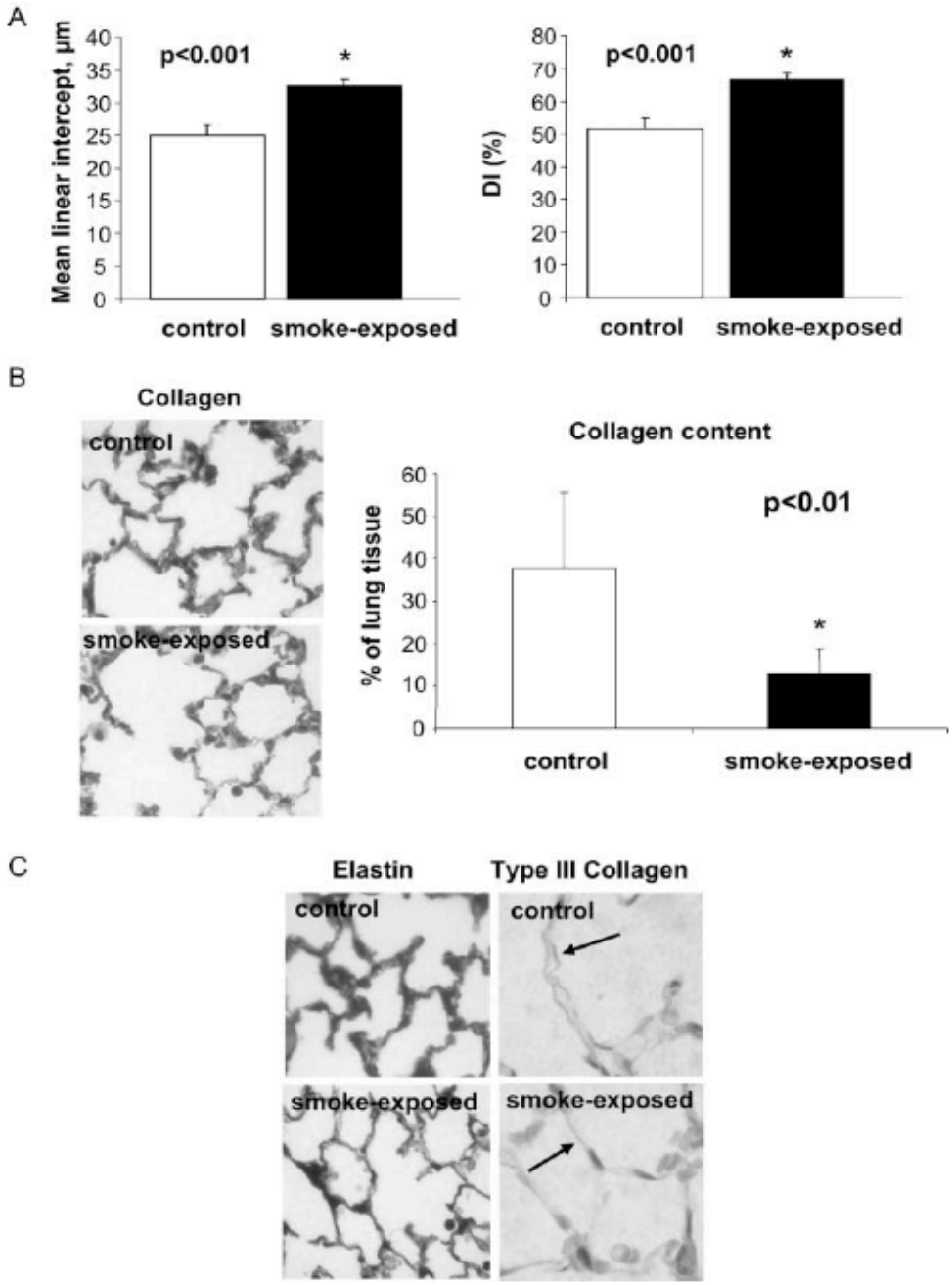

FIGURE 3 Emphysematous changes and decreased extracellular matrix content in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks. (*A*) The mean linear interecept (MLI) and destructive index (DI) were measured in H&E-stained lung tissue sections from control and smoke-exposed guinea pigs. A statistically significant increase in MLI was observed in the lungs from guinea pigs exposed to cigarette smoke for 12 weeks (32.5 μm versus 25 μm for the controls; $P < .001$). A destructive index (DI) analysis exhibited a similar pattern (67% versus 51% for the controls; $P < .001$). (*B*) Lung sections of control and smoke-exposed animals were stained for collagen (Masson's trichrome) and staining was quantified by videomicroscopy. A decrease in collagen content in the lungs of smoke-exposed animals was observed. (*C*) Lung sections of the guinea pigs were stained for elastin (Elastica Van Gieson) and type III collagen. Reduction of elastin and type III collagen was demonstrated in the lungs of guinea pigs exposed to cigarette smoke for 12 weeks.

matrix in the guinea pig, causing a decrease in elastin and interstitial collagen as is seen in mouse models.

Increased Cathepsin K Activity in the Lungs of Smoke-Exposed Guinea Pigs

Proteases play a critical role in the pathogenesis of smoke-induced emphysema. To detect the activity of collagenolytic MMPs in the lungs of smoke-exposed guinea pigs, we performed quantitative RT-PCR analysis. MMP expression (Mcol-A, MMP-1, MMP-8, MMP-12, MMP-13, and MMP-14) at the mRNA level was unchanged (data not shown). As the complete sequences of guinea pig MMPs have not been determined, we tested assays that have been developed based on the human and mouse sequences. To investigate the effect of cigarette smoke on the activity of other collagenalytic enzymes in the guinea pig lung, we measured the activities of cathepsins K, S, and L in lung lysates using a specific activity assay. The activity of cathepsin K was significantly higher in the lung extracts of smoke-exposed guinea pigs compared with control animals (Figure 4*A*). To determine the expression pattern of cathepsin K in the guinea pig lung, we performed immunostaining, which exhibited an increase in signal intensity in the lungs of smoke-exposed guinea pigs. The stronger signal localized to the alveolar macrophages when compared to the epithelial cells (Figure 4*B*). Furthermore, an increase in cathepsin S activity was detected in the lung lysates of smoke-exposed guinea pigs, though the difference between the smoke-exposed and control group was not significant (Figure 4*A*). The acitivity of cathepsin L was unchanged for both groups (Figure 4*A*).

Increase in Cathepsin K Expression in the Lungs of the Patients With Emphysema

Our data suggest that cathepsin K is involved in the destruction and remodeling processes of the lung in the guinea pig model of smoke-induced emphysema. To determine whether cathepsin K levels were altered in the lungs of patients with emphysema, we analyzed the lysates of the lungs of normal and emphysema patients by Western blotting. A marked increase in cathepsin K expression was seen in the lungs of patients with emphysema compared to normal subjects (Figure 5).

DISCUSSION

In our in vivo guinea pig model of smoke-induced emphysema, exposure to cigarette smoke caused lung inflammation, with a significant increase in the recruitment of alveolar macrophages. After 12 weeks of exposure, the lungs exhibited emphysematous changes, with dilation of the alveolar wall

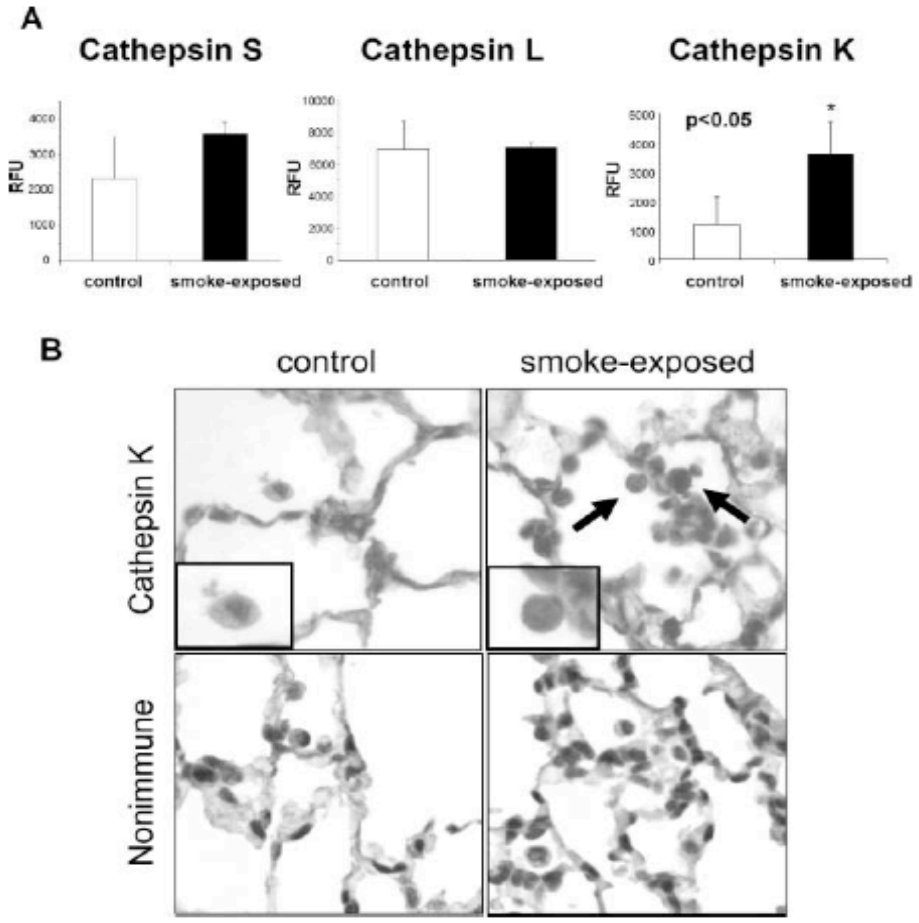

FIGURE 4 Increased cathepsin K activity in guinea pig lung after smoke exposure. (*A*) Cathepsin K, S, and L activities in lung tissue lysates were assayed with specific fluorescent substrates. A significant increase ($P < .05$) in the activity of cathepsin K was demonstrated in the lung lysates of smoke-exposed animals. (*B*) Cathepsin K was detected by immunohistochemistry in the alveolar macrophages (*arrows*) of the guinea pigs exposed to cigarette smoke. Nonimmune rabbit serum was used as a negative control.

and loss of the extracellular matrix. We also observed increased activation of 2 major MAP kinases pathways, ERK and JNK, due to smoke exposure. Although collagenolytic MMPs were not significantly modulated by smoke, elevated cathepsin K activity was present in the lungs of smoke-exposed animals. Cathepsin K is a potent elastase and collagenase, and therefore likely contributes to the observed loss of lung extracellular matrix in this model.

Multiple studies indicate that lung inflammation is a hallmark of smoke-induced emphysema [16–18]. Our data demonstrate that in response to cigarette smoke exposure, guinea pigs develop a marked increase in lung inflammation characterized by a significant elevation in the number of alveolar macrophages. In the guinea pig model, elevated number of macrophages correlated with increased MMP-9 in bronchoalveolar lavage fluid. MMP-9 is a marker of lung inflammation and is expressed mainly in macrophages

[14]. After 12 weeks of smoke exposure, guinea pigs developed emphysematous changes, with the characteristic destruction of the lung extracellular matrix and abnormal enlargement of the airspaces. Smoke-exposed animlas exhibited a 30% increase in the mean linear intercept. In contrast, mice have been shown to be much more resistant to the development of smoke-induced emphysema. Consistent with these findings, we have demonstrated that mice exposed to cigarette smoke in the identical fashion as the guinea pigs in this study manifested a 16% increase in the mean linear intercept but after 1 year of exposure [19]. Our data therefore indicate that guinea pigs are more susceptible to emphysema formation in response to chronic smoke exposure. Although presence of apoptotic cells have been reported in mice post smoke exposure, our laboratory did not observe any evidence of apoptosis in the lungs of mice [19] and in the present guinea pig model after exposure to smoke.

The investigation of the downstream MAP kinase signaling pathways in the lungs of smoke-exposed guinea pigs provides insight into the molecular

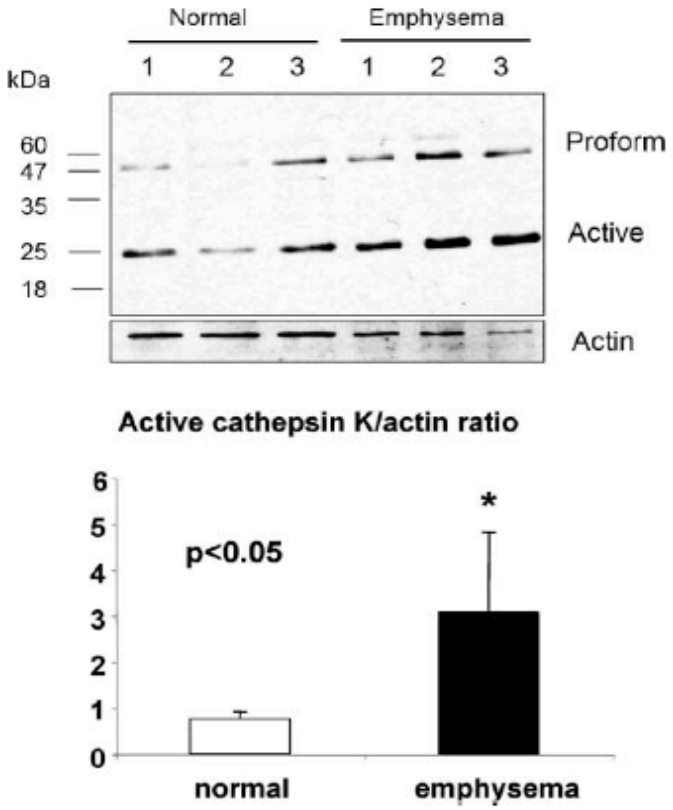

FIGURE 5 Up-regulation of cathepsin K in the lungs of patients with emphysema. Lung lysates were analyzed by Western blotting using a monoclonal antibody. The amount of cathepsin K was elevated in emphysematous lung samples compared with normal lungs ($n = 3$ for each condition). Both the pro- (40-kDa) and active (25-kDa) forms of cathepsin K were detected. Active cathepsin K/actin ratio was determined by densitometric analysis ($n = 3$ for normal lungs and $n = 6$ for emphysematous lungs) using Image Pro 4.5 software.

mechanisms of smoke-induced emphysema. Our laboratory has demonstrated that ERK is activated in the lungs of patients with emphysema and induces the expression of MMP-1 in small airway epithelial cells [20], a protease that causes emphysema in transgenic mice [6]. It has therefore been hypothesized that smoke-induced phosphorylation of mitogen-activated protein kinases play an important role in the deregulation of the balance between proteases and antiproteases in the lung extracellular matrix. In the guinea pig model, we revealed an increase in ERK and JNK phosphorylation due to smoke exposure. The activation of ERK and JNK has already been demonstrated in rodent models of emphysema [20, 21]. As demonstrated in the rodent model, smoke activation of both ERK and JNK in the lung of the guinea pig likely affects the inflammatory process leading to the development of emphysema.

In the guinea pig model, the development of emphysema was accompanied by increased destruction of the components of lung extracellular matrix. The marked destruction of pulmonary collagen and elastin is a hallmark of smoke-induced emphysema. In transgenic mice overexpressing MMP-1, the development of emphysema was attributed to the loss of type III collagen [8], suggesting that, in humans, the disruption of this fibrillar collagen is a crucial event leading to emphysema. A striking reduction of the type III collagen in the lungs of smoke-exposed guinea pigs was also observed in our study consistent with what is seen in the mouse model [8]. The loss of elastin and type III collagen can contribute to the change in compliance seen in the guinea pig smoke exposure model.

Although MMP-1 is overexpressed in human emphysema [4] and augmented expression of MMP-13 has been seen in response to cigarette smoke in murine lungs [22], we did not detect any significant up-regulation of collagenolytic MMPs (MMP-1, -8, -13, -14, and McolA) in the lungs of smoke-exposed guinea pigs. Interestingly, Selman and colleagues detected MMP-1 mRNA in alveolar macrophages and epithelial cells in the damaged lungs of the guinea pigs exposed to cigarette smoke [23]. These authors used human MMP-1 cDNA as a probe in their experiments because the cDNA sequence of guinea pig MMP-1 was not yet determined at that time. However, in the present study, we performed RT-PCR using primers specific to guinea pig MMP-1 [24], and we did not detect MMP-1 mRNA expression in the lungs of control and smoke-exposed animals. Therefore, because collagenolytic MMPs were not significantly modulated in the lungs of guinea pigs exposed to smoke, the observed decrease in collagen and elastin content likely results from the up-regulation of other classes of proteolytic enzymes.

It has been demonstrated that cathepsins degrade major proteins of extracellular matrix and limit the release of newly synthesized collagen and elastin [25, 26]. In addition, Zheng and colleagues showed that IL-13–induced activity of cathepsins such as B, S, L, H, and K leads to the development of emphysema in the adult murine lung [9]. In our study,

we demonstrated increased activity of cathepsin K in the lungs of smoke-exposed guinea pigs, which likely contributes to the development and progression of cigarette smoke-induced emphysema. Specific enzymatic assays of lung extracts revealed a significant increase in the activity of cathepsin K in the smoke-exposed guinea pigs. Cathepsin K is a cysteine protease, which possesses both collagenase and elastase activities, capable of degrading fibrillar collagen at several sites, contrary to MMPs, which cleave collagen at one specific site [3]. Therefore, we believe that increased activity of such potent proteolytic enzyme is likely responsible for the development of emphysema in the guinea pig lung. The expression of cathepsin K was detected mainly in the alveolar macrophages of the smoke-exposed animals, whereas collagenolytic MMP-1 expression in humans was identified in the parenchymal epithelial cells. Additionally, Mercer and colleagues demonstrated that cigarette smoke induces MMP-1 expression in the small airway epithelial cells [20]. Moreover, the elevated expression of cathepsin K was detected in the human lung extracts of patients with emphysema, confirming the clinical relevance of cathepsin K in human disease. Therefore, this finding is noteworthy for its contribution to the understanding of the pathogenesis of emphysema, suggesting that dysregulated activity of cathepsins as well as MMPs might lead to the irreversible changes in the lung structure.

In addition to cathepsin K, cathepsin S activity was also detected in the lungs of smoke-exposed guinea pigs, but its modulation by smoke was not statisticaly significant. A recent study [27] showed an enhanced expression of cathepsin S in mice after cigarette smoke exposure. Lack of neutrophils, the absence of MMP-13 and cathepsin S up-regulation, together with an increased susceptibility to emphysema in guinea pigs suggest that the mechanisms involved in the alveolar wall disruption differ between small rodents and guinea pigs.

The present study suggests that cathepsin K may contribute to the remodeling of the lung extracellular matrix after exposure to smoke. Cathepsins have been shown to be regulated by MAP kinases in several cell lines in culture [28]. Therefore, the modulation of ERK and JNK pathways due to smoke may be responsible for the up-regulation of cathepsin K in the lungs. Further studies are needed to understand the precise role and regulation of cathepsin K during emphysema formation in this animal model and to identify if similar regulation occurs in the human disease.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 3

The development of emphysema in murine models of atherosclerosis

Abstract

Smokers with airflow obstruction have an increased risk of atherosclerosis, but the relationship between the pathogenesis of these diseases is not well understood. To determine if hypercholesterolemia alters lung inflammation and emphysema formation, we examined the lung phenotype of two hypercholesterolemic murine models of atherosclerosis at baseline and on a high-fat diet and explored the mechanism of lung injury. Airspace enlargement developed in the lungs of *Apoe*$^{-/-}$ mice exposed to a Western-type diet for 10 weeks. An elevated number of macrophages and lymphocytes accompanied by an increase in matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-12 (MMP-12) expression was observed in the lungs of *Apoe*$^{-/-}$ mice on a Western-type diet. In contrast, *Ldlr*$^{-/-}$ mice exposed to a Western-type diet for 10 weeks did not exhibit lung destruction or inflammatory changes. Most importantly, we revealed augmented expression of the downstream targets in the toll-like receptor (TLR) pathway, interleukin-1 receptor-associated kinase 1 (IRAK1) and granulocyte colony-stimulating factor (G-CSF), in the lungs of *Apoe*$^{-/-}$ mice. In addition, we demonstrated overexpression of MMP-9 in the *Apoe*$^{-/-}$ macrophages treated with TLR4 ligand, suggesting that emphysema in these mice is a result of the activation of the TLR pathway secondary to hyperlipidemia and abnormal cholesterol efflux. Our findings indicate that hyperlipidemia and abnormal cholesterol efflux lead to increased systemic inflammation with subsequent lung damage and emphysema formation in *Apoe*$^{-/-}$ mice.

Introduction

Emphysema is a chronic obstructive pulmonary disease characterized by the abnormal destruction of alveolar walls accompanied by the enlargement of airspaces (Sharafkhaneh et al., 2008). Today more than twelve million people in the United States are diagnosed with this condition (Krishnan, 2010). The incidence of emphysema in cigarette smokers is much higher than in non-smokers and the association between the progression of the disease and the degree of cigarette smoking has been documented by several studies (Vestbo et al., 2006; Higgins, 1991). Cigarette smoke exposure causes increased inflammation (Grumelli et al., 2004), protease/antiprotease imbalance (Shapiro, 1995), apoptosis (Tuder et al., 2003) and oxidative stress (Rahman et al., 1996). These processes are believed to contribute to the alveolar destruction. It has also been shown that smokers face an increased risk for numerous diseases, including atherosclerosis. Cigarette smoke raises the levels of oxidized low-density lipoprotein (LDL) cholesterol and damages vessel endothelium leading to the development of atherosclerosis (Stokes, 1990). Of note, smokers with airflow limitation have more prominent atherosclerosis than smokers with normal lung function, suggesting a link between atherosclerosis and obstructive lung disease (Iwamoto et al., 2009).

One of the major hallmarks of emphysema and atherosclerosis is inflammation originating from the infiltration of macrophages and lymphocytes into the airway and vessel wall, respectively. Atherosclerotic lesions reveal increased numbers of lipid-laden macrophages (Hansson, 2005). Likewise, smokers with airflow limitations exhibit an increase in the number of macrophages and T-lymphocytes in the lung (Wouters, 2005). The presence of these inflammatory cells correlates with the development and

progression of emphysema (Larsson, 2007). Animal models of atherosclerosis and emphysema demonstrate similar inflammatory profiles (Stoll et al., 2006; Taraseviciene-Stewart et al., 2008). Therefore, we examined the lungs of two murine models of atherosclerosis in order to assess the potential consequences of hyperlipidemia on lung structure.

The most widely used murine models for atherosclerosis are *Apoe*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice, which both develop hypercholesterolemia (Veniant et al., 2001; Knowles et al., 2000). Under normal conditions, ApoE accepts cholesterol from cells and transports it back to the liver where it can be excreted. The loss of ApoE and the LDL receptor result in hypercholesterolemic states due to the impaired lipoprotein production and metabolism (Veniant et al., 2001; Knowles et al., 2000). One of the critical roles of ApoE is to promote cholesterol efflux from macrophages (Basu et al., 1981; Mazzone et al., 1994). Deficiency of endogenous ApoE expression leads to the deleterious effects of cholesterol-overloaded macrophages, which are also known as foam cells (Reddick et al., 1994). Accumulation of oxidized LDL in macrophages of the plaque has been demonstrated to stimulate the secretion of various cytokines and proteases, which leads to the degradation of the components of the extracellular matrix (Shaw, 2004). The goal of this study was to examine the lung phenotype in *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ to determine if the hypercholesterolemia and abnormal cholesterol efflux impacted on the extracellular matrix of the lung.

Materials and Methods

Animal experiments

Two murine models of atherosclerosis were used to study the effect of hypercholesterolemia on the lung structure. The first group included 8-week-old female *Apoe*$^{-/-}$ mice (n=6) subjected to a Western diet for 10 weeks, compared to *Apoe*$^{-/-}$ mice on a chow diet (n=6) and *Apoe*$^{+/+}$ controls (n=6). In the second group of animals, the *Ldlr*$^{-/-}$ model of atherosclerosis was used to observe the effect of the diet on the lungs. *Ldlr*$^{-/-}$ female mice were exposed to a Western diet (n=6) or chow (n=6) for 10 weeks. Female mice were selected due to their increased susceptibility to the atherosclerotic plaque formation. Mice were fed an atherogenic high-fat Western-type diet (20% protein, 50% carbohydrate, 21% fat, 0.21% cholesterol; Research Diets, NJ, D12079B). All mice were on a C57BL6/J background obtained from Jackson Laboratories. Animals were housed at Columbia University Medical Center according to animal welfare guidelines. Food and drinking water were provided ad libitum. All animal studies were approved by the Columbia University Institutional Animal Care and Use Committee.

Cell Culture

Peritoneal macrophages were obtained from *Apoe*$^{-/-}$ and *Apoe*$^{+/+}$ mice injected intraperitoneally with thioglycollate. The macrophages were cultured in DMEM containing 5% FBS (fetal bovine serum) for 24 hours and subsequently treated with various toll-like receptor (TLR) ligands. TLR2 [peptidoglycan (PGN), 2μg/ml], TLR3 [polyinosine-polycytidylic acid (poly(I:C), 2.5μg/ml], and TLR4 [lipid A, the active

component of lipopolysaccharide, 100ng/ml] ligands were used for the experiment. Macrophages were also treated with TLR4 ligand and oxidized LDL (100μg/ml).

Histology and immunohistochemistry

After exposure to the Western or chow diet, mice were sacrificed by carbon dioxide inhalation. The trachea was cannulated with a 16g argon catheter secured with a silk suture. The lungs were lavaged first with PBS (1ml), to collect bronchoalveolar lavage (BAL) fluid, then infused with 10% formalin for 24 hours. Tissues were embedded in paraffin and sectioned (6 μm). Sections were stained with hematoxylin and eosin (H&E) for histological analysis and quantification of macrophages. Morphometric analysis of the H&E stained lungs of 6 mice in each group was performed as previously described (Foronjy et al., 2006). Morphometric assessment was conducted to determine the average distance between alveolar walls (mean linear intercept), the fractional volume of parenchyma tissue per lung, and the alveolar surface area per unit volume. Ten histological fields (400X magnification) were analyzed from at least two separate sections from each mouse (n=6 in each group) to calculate morphometric parameters.

Western Blotting

Freshly dissected lungs of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (10 mg) were homogenized in 1ml of protein lysis buffer (PBS containing Triton X-100 0.1%), and centrifuged (14000g for 10 minutes). Fifty μg of the lung lysates of each group were subjected to Western Blot analysis. Rabbit polyclonal antibodies against phospho- (ph-) ERK, ph-JNK (Cell

Signaling) and IRAK-1 (Santa Cruz) were used, following the manufacturer's instructions.

Zymography

Zymography was performed to detect proteases having gelatinolytic activity, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) as previously reported (Heussen et al., 1980). Ten mg of gelatin (Sigma-Aldrich) were dissolved in 10 ml of a SDS-polyacrylamide gel. After loading and migration of the protein samples, the gel was washed three times for 20 minutes in Triton X-100 2.5%, to remove the SDS. The gel was then placed in 20 ml of a buffer optimal for MMP activity (Tris-HCl 50 mM; Triton X-100 1% vol/vol; CaCl2 5 mM; ZnCl2 1 uM; Sodium azide 0.05 % w/v; pH7.4). During the overnight incubation at 37C, gelatinolytic enzymes present in the gel digested the co-polymerized substrate. After staining with Coomassie blue, the gel appeared blue except where the substrate was digested, forming clear bands. This technique is very sensitive for the detection of MMP-9 and MMP-2, two highly active gelatinases.

Quantitative RT-PCR

Total RNA was extracted from two specimens of lung tissue 0.3cm^3 in size with the use of a RNeasy kit (Qiagen). TaqMan gene expression assays were performed to assess gene-transcript levels with the use of an ABI Prism® 7900HT Sequence Detection System (Applera Corporation, Foster City, CA). Primer and probe sets were purchased from ABI and included the following: MMP-9 (Mm00442991_m1) and matrix metalloproteinase-12 (MMP-12) (Mm00500554_m1). Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was used as the housekeeping gene. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed on lung tissue from four animals in each group. To calculate the relative quantity (RQ) of MMP-9 and MMP-12, we used the $2^{-\Delta\Delta CT}$ method implemented in the software. The data are shown as the fold change in gene expression normalized to the endogenous reference gene GAPDH and relative to the controls.

Statistical analysis

Data are shown as mean ±SD. Unpaired two-tailed Student's t-test analysis was performed to determine statistical significance ($p<0.05$).

Results

Development of emphysema in *Apoe*$^{-/-}$ mice exposed to an atherogenic high-fat diet

Emphysema is defined as a disease characterized by abnormal permanent enlargement of airspaces distal to the terminal bronchiole and accompanied by destruction of alveolar walls (Snider et al., 1985). The effect of hypercholesterolemia on the development of emphysema in the *Apoe*$^{-/-}$ mice subjected to an atherogenic high-fat diet for 10 weeks was estimated by measuring the mean linear intercept (MLI). Morphometric analysis demonstrated that *Apoe*$^{-/-}$ mice fed a high-fat diet for 10 weeks developed statistically significant airspace enlargement compared with their controls (41.3μm vs. 32.6μm for the controls, $p<0.0005$) **(Table 1)**. Interestingly, *Apoe*$^{-/-}$ mice fed a chow diet for 10 weeks did not develop airspace enlargement and their MLI was lower compared to the MLI of *Apoe*$^{-/-}$ mice fed a high-fat diet for 10 weeks (36.8μm vs. 41.3μm, $p<0.04$). *LDLr*$^{-/-}$ mice subjected to a high-fat diet for 10 weeks did not exhibit emphysematous changes in their lungs **(Figure 1).** However, *LDLr*$^{-/-}$ mice maintained on a high-fat diet for 18 weeks developed significant airspace enlargement (29.3μm vs. 44.8μm, $p<0.0009$) **(Figure 2)**. The measurements of alveolar surface area in the lungs of *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice confirmed the assessment of MLI **(Table 1).** *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice had similar cholesterol levels when fed a Western-type diet (an average of 1118 mg/dl vs. 1167 mg/dl). However, higher cholesterol levels have been observed in *Apoe*$^{-/-}$ mice compared to *LDLr*$^{-/-}$ during the period preceding the high-fat diet (an average of 818 mg/dl vs. 358 mg/dl) **(Table 1).**

Inflammatory response to hypercholesterolemia in the lungs of *Apoe*$^{-/-}$ mice

To evaluate the impact of hypercholesterolemia on the inflammation in the lungs of *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice, macrophages and lymphocytes were quantified in tissue sections of mice exposed to a Western-type diet for 10 weeks and their wild-type controls. An increased number of macrophages was observed in the lungs of *Apoe*$^{-/-}$ mice fed a Western-type diet for 10 weeks (7.72±1.33 macrophages per mm^2 for the controls vs. 10.23±1.04 macrophages per mm^2 for the *Apoe*$^{-/-}$ mice, $p<0.005$) **(Figure 2A).** Additionally, *Apoe*$^{-/-}$ mice exhibited an increased number of lymphocytes in their lungs. **(Figure 2B).** The number of macrophages was not significantly different in the lungs of *LDLr*$^{-/-}$ mice fed a Western-type and chow diet for 10 weeks.

Increased expression of MMP-9 and MMP-12 in the lungs of *Apoe*$^{-/-}$ mice subjected to a Western-type diet

Matrix metalloproteinases (MMPs) play a crucial role in the pathogenesis of emphysema (Lemaitre et al., 2006; Lagente et al., 2005; Imai et al., 2001). It has been demonstrated that overexpression of MMP-9 in macrophages leads to the development of spontaneous emphysema in mice (Foronjy et al., 2008), while loss of MMP-12 protects mice from the development of emphysema (Hautamaki et al., 1997). Therefore, we performed zymography to determine the activity of MMP-9 in the lungs of *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice subjected to a Western-type diet for 10 weeks. Increased activity of MMP-9 was observed in the BAL fluid from *Apoe*$^{-/-}$ mice **(Figure 3A)**. MMP-2 activity was detected in the BAL fluid of Apoe$^{-/-}$ and Apoe$^{+/+}$ mice but its levels were not altered. Small increase in MMP-9 activity was also detected in the BAL fluid from *LDLr*$^{-/-}$ mice fed a Western-

type diet for 10 weeks **(Figure 3B)**. Quantitative RT-PCR analysis confirmed elevation of MMP-9 on mRNA level in the lungs of *Apoe*$^{-/-}$ mice **(Figure 3C).** RT-PCR analysis revealed augmented expression of MMP-12 at the mRNA level in the lungs of *Apoe*$^{-/-}$ mice (n=4) compared with controls (n=4) **(Figure 3C).**

Activation of TLR signaling pathway in response to hypercholesterolemia in the lungs of *Apoe*$^{-/-}$ mice

The Toll-like receptor (TLR) pathway is activated in response to the accumulation of cholesterol in macrophages in *Apoe*$^{-/-}$ mice linking hyperlipidemia and TLR signaling (Björkbacka et al., 2004). Interleukin-1 receptor activated kinase (IRAK) is a key regulator in the signaling pathway of TLRs. Once activated IRAK initiates a cascade of signaling events ultimately leading to the induction of inflammatory genes such as granulocyte colony-stimulating factor (G-CSF) (Bozinovski et al., 2002; Szczepanski et al., 2009). To evaluate the involvement of the TLR pathway in the development of emphysema in *Apoe*$^{-/-}$ mice, we analyzed the expression of TLR4 and its downstream targets IRAK1 and G-CSF in the lungs. We did not observe changes in relative mRNA expression of TLR4 (RQ=1.0 ± 0.2 for Apoe$^{+/+}$ vs 0.6 ± 0.3 for *Apoe*$^{-/-}$, P=0.1; n=4 in each group). However, the analysis of IRAK1 and G-CSF expression revealed their upregulation in the lungs of *Apoe*$^{-/-}$ mice fed a Western-type diet **(Figure 4A and 4B)**, indicating that the TLR pathway is activated in these mice.

TLR signaling mediates MMP-9 expression induced by hypercholesterolemic diet in Apoe$^{-/-}$ mice

The Toll-like receptor signaling pathway is involved in various cholesterol-induced processes and links hypercholesterolemia with overexpression of inflammatory and chemokine genes. In addition, TLR signaling regulates protease expression in peritoneal macrophages (Sun et al., 2009). To determine whether *Apoe*$^{-/-}$ macrophages were more responsive to TLR activation, peritoneal macrophages from *Apoe*$^{-/-}$ mice and their controls were treated with activators of various TLR ligands. TLR4 increased mRNA expression of MMP-9 by 8-fold in *Apoe*$^{-/-}$ macrophages and by 4-fold in wild-type macrophages **(Figure 4C).** Interestingly, TLR2 and TLR3 ligands did not exhibit any effect on MMP-9 expression in *Apoe*$^{-/-}$ and wild-type macrophages, suggesting that the macrophage responsiveness was TLR4 specific **(Figure 4C).**

Activation of ERK and JNK, downstream regulators of TLR signaling pathway, in the lungs of *Apoe*$^{-/-}$ mice subjected to a Western-type diet

Mitogen activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK) are activated by TLR signaling pathway (Hirata et al., 2008). Phosphorylation of ERK and JNK results in the activation of transcription factors regulating inflammatory genes (Hodgkinson et al., 2008). Our laboratory has demonstrated that phosphorylated ERK is increased in the lungs of patients with emphysema (Mercer et al., 2004). To determine if *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice exposed to a Western-type diet have increased levels of phospho-ERK and phospho-JNK in the lung, we analyzed lung lysates by Western blotting. This analysis revealed elevated

expression of phospho-ERK and phospho-JNK in the lungs of *Apoe*$^{-/-}$ mice **(Figure 5A and 5B)**. Interestingly, we also observed augmented expression of phospho-ERK in the lungs of *LDLr*$^{-/-}$ mice suggesting that activation of ERK potentially occurs early under hypercholesterolemic conditions **(Figure 5C)**. In addition, we revealed that the activation of ERK in *Apoe*$^{-/-}$ macrophages by oxidized LDL was further increased by the addition of TLR4 ligand **(Figure 5D)**.

Discussion

In the present study, *Apoe*$^{-/-}$ mice subjected to an atherogenic Western-type diet for 10 weeks developed emphysematous changes with enlargement of airspaces and destruction of alveolar walls. In contrast, *Apoe*$^{+/+}$ and *Apoe*$^{-/-}$ on a chow diet and *LDLr*$^{-/-}$ mice on an atherogenic diet did not manifest emphysematous changes after 10 weeks. Hypercholesterolemia in *Apoe*$^{-/-}$ mice resulted in lung inflammation with a significant increase in the number of macrophages and lymphocytes. TLR pathway activation and augmented expression of two major elastinolytic proteases (MMP-9 and MMP-12) was observed in the lungs of the hypercholesterolemic *Apoe*$^{-/-}$ mice. These potent proteolytic enzymes likely contributed to the observed destruction of lung extracellular matrix in the *Apoe*$^{-/-}$ mice. We also demonstrated that the macrophages from *Apoe*$^{-/-}$ mice were sensitive to TLR4 activation and hypothesize that activation of the TLR4 pathway secondary to hypercholesterolemia is likely responsible for the increased inflammation in the lung.

Studies have suggested that obstructive pulmonary disease is a systemic disease rather than an independent disease state (Fabbri et al., 2007). Atherosclerosis is one of the leading causes of mortality in COPD (Anthonisen et al., 2002). The major hallmark of both pathologies is the presence of chronic inflammation with the recruitment of macrophages and lymphocytes (Back, 2008). It has been demonstrated that murine models of smoke-induced emphysema exhibit a marked increase in the number of macrophages and neutrophils and have augmented myeloperoxidase activity (Foronjy et al., 2006). Additionally, mouse models of COPD exhibit expansions of T-cells in their lungs in response to chronic cigarette smoke exposure (Motz et al., 2008). Interestingly,

Apoe$^{-/-}$ mice fed only a Western-type diet for 10 weeks had both an increase in the number of pulmonary macrophages and lymphocytes. These aggregations of lymphocytes are likely secondary to changes in lipid metabolism present in *Apoe*$^{-/-}$ mice. In contrast, in *LDLr*$^{-/-}$ mice, emphysematous changes were observed only after 18 weeks on a high-fat diet. Despite exhibiting elevated cholesterol, after 10 weeks of a high-fat diet *LDLr*$^{-/-}$ mice did not develop emphysema and exhibited less extensive pulmonary inflammation compared to *Apoe*$^{-/-}$ mice. Increased susceptibility to emphysema formation in *Apoe*$^{-/-}$ mice compared to *LDLr*$^{-/-}$ mice may be attributed to differences in cholesterol levels and in cholesterol efflux. Notably, on a regular chow diet, *Apoe*$^{-/-}$ mice have higher cholesterol levels compared to *LDLr*$^{-/-}$ mice (an average of 818 mg/dl vs. 358 mg/dl on a chow diet). *Apoe*$^{-/-}$ macrophages also exhibit abnormal cholesterol efflux, which leads to a pathological lipid accumulation in these cells. This defect in cholesterol transport is caused by the deficiency of ApoE, which mainly functions to promote macrophage cholesterol efflux (Tall et al., 2002; Zhu et al., 1998). Interestingly, *Abca1*$^{-/-}$ and *Abcg1*$^{-/-}$ mice manifesting abnormal cholesterol efflux exhibit striking pulmonary inflammation (Baldan et al., 2008; Bates et al., 2005). In addition, increased inflammatory gene expression via TLR4 signaling was observed in *Abca1*$^{-/-}$ and *Abcg1*$^{-/-}$ macrophages, suggesting that there is a link between cholesterol efflux, lung inflammation and TLR4 signaling (Yvan-Charvet et al., 2008). Therefore, higher cholesterol levels and possibly impaired cholesterol efflux likely explain why *Apoe*$^{-/-}$ mice are more susceptible to emphysema compared to *LDLr*$^{-/-}$ mice.

In the present study we provide evidence for the role of TLR signaling in the development of emphysema in *Apoe*$^{-/-}$ mice. The Toll-like Receptor signaling pathway

plays a crucial role in the regulation of the immune response in atherosclerosis and in various lung-associated pathologies (Basu et al., 2004). It has been demonstrated that ablation of IL-1R1 and MyD88, downstream regulators of TLR4 signaling, in mice prevents lung inflammation and emphysema (Couillin et al., 2009). Furthermore, smoke-exposed mice with targeted ablation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a potent regulator of ROS production, exhibit pulmonary inflammation, emphysematous changes and enhanced activation of the TLR4 signaling pathway (Yao et al., 2008). The TLR signaling pathway also plays an essential role in the development of atherosclerosis and is regulated by oxidized LDL (Choi et al., 2009). In addition, it has been reported that oxidized LDL-induced foam cell formation is positively associated with increased expression of TLR2 and TLR4 (Michelsen et al., 2004; Xu et al., 2001), with TLR4 reducing the expression of the genes involved in cholesterol transport and metabolism causing a pathological lipid accumulation in macrophages (Castrillo et al., 2003).

Numerous studies have shown that activation of TLR2 or TLR4 leads to the recruitment of IRAK1, IRAK4 and TRAF6 (Harju et al., 2001). This association can result in the activation of ERK and JNK, MAP kinases involved in TLR signaling. The present study provides evidence for TLR4 signaling activation in the lungs of *Apoe*$^{-/-}$ mice. Activation of this pathway is indicated by analysis of downstream targets in the lung, and by *ex vivo* experiments on elicited macrophages. In lungs of *Apoe*$^{-/-}$ mice, we found upregulation of IRAK1 and activation of ERK and JNK MAP kinases, which are downstream targets of TLR4. We have shown previously that ERK is activated in the lungs of patients with emphysema and is likely a key regulator of MMP expresssion in

this disease (Mercer et al., 2004). The upregulation of G-CSF, a known dowstream target of TLR, was also observed in the lungs of *Apoe*$^{-/-}$ mice, which probably accounts for the significant increase in macrophage infiltration seen in *Apoe*$^{-/-}$ mice compared to the *LDLr*$^{-/-}$ animals.

Increased expression of MMP-9 and MMP-12 was demonstrated in the lungs of *Apoe*$^{-/-}$ mice. An imbalance of proteases and antiproteases in human lung and endothelium is known to contribute to the development of emphysema and atherosclerosis, respectively (Lemaitre et al., 2006; Abboud et al., 2008; Zadelaar et al., 2007). Several animal studies have confirmed the important role for MMPs in the pathogenesis of emphysema and atherosclerosis. We recently demonstrated that overexpression of MMP-9 in macrophages of transgenic mice caused spontaneous emphysema due to elastin degradation, while MMP-12 (metalloelastase) - deficient mice are protected from emphysema formation after smoke exposure (D'Armiento et al., 1992; Foronjy et al., 2008; Hautamaki et al., 1997). Therefore, upregulation of MMP-9 and -12 in the lungs of *Apoe*$^{-/-}$ mice ultimately contributes to the observed emphysematous changes, through a disruption of the lung extracellular matrix.

Since macrophages are a major source of MMP-9, we treated elicited macrophages from *Apoe*$^{-/-}$ mice with various TLR ligands and demonstrated that TLR4 ligand, but no other ligand, augmented MMP-9 expression. In addition, we observed that, in *Apoe*$^{-/-}$ macrophages, the activation of ERK due to oxidized LDL was increased by TLR4 ligand, indicating a role for TLR4 signaling in mice with abnormal cholesterol efflux. These findings suggest that TLR4 signaling is responsible for ERK activation and MMP-9 upregulation in the lungs of *Apoe*$^{-/-}$ mice, contributing to the observed

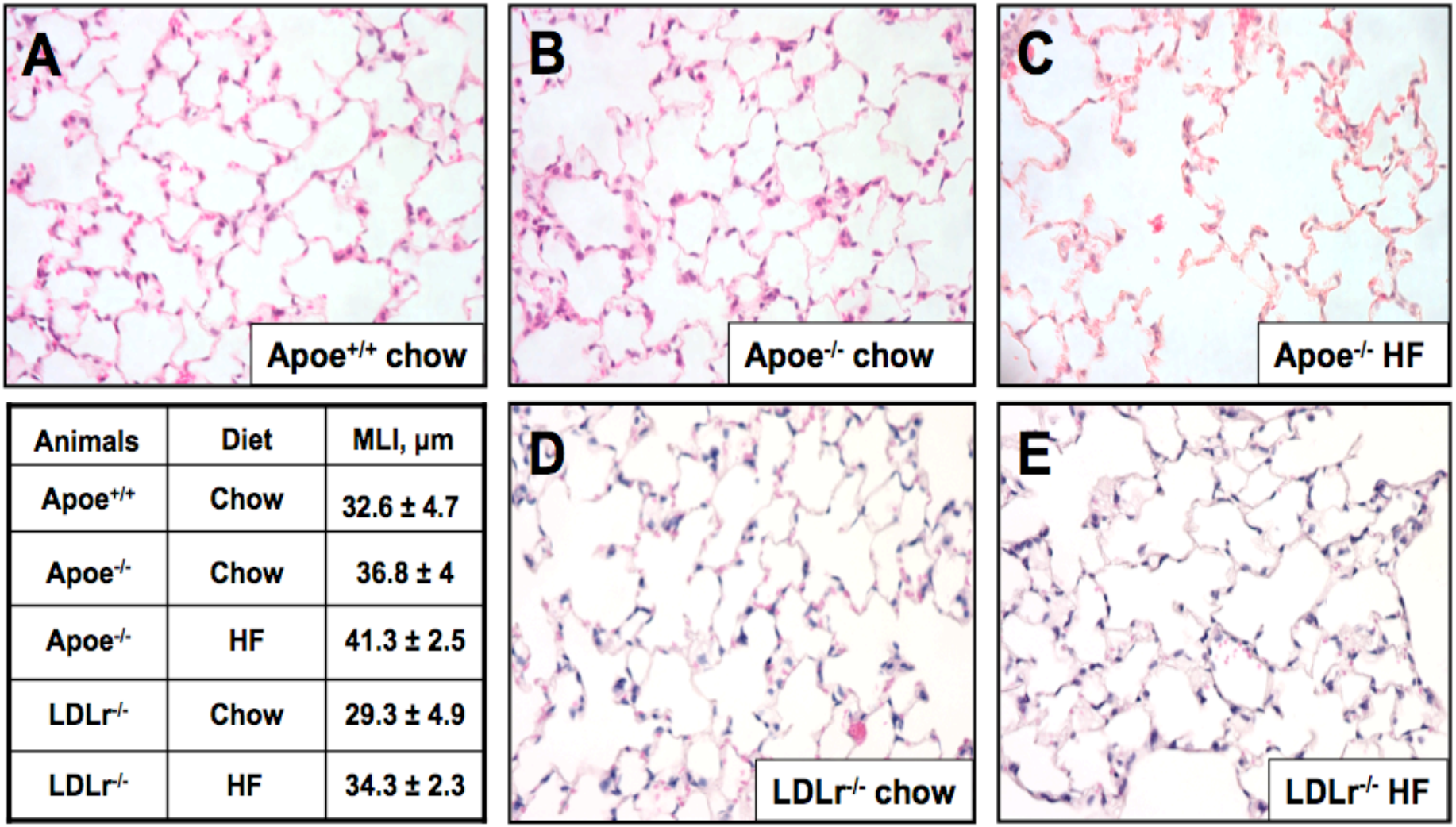
emphysematous changes. In future experiments, the use of a conditional, macrophage-specific TLR4 knockout model crossed into the Apoe-deficient background will provide direct evidence for the involvment of this receptor in emphysema formation.

In summary, our study indicates that, in *Apoe*$^{-/-}$ mice fed a Western-type diet, severe systemic hypercholesterolemia accompanied by the abnormal cholesterol efflux induces pulmonary inflammation through a TLR4/ERK/MMP cascade, affecting the integrity of the lung. The synergistic effect of dyslipidemia and defective cholesterol efflux observed in atherosclerosis may also contribute to emphysema, providing a mechanistic link between atherosclerosis and emphysema. Our findings also add to the increasing evidence for the role of TLR signaling in lung diseases and in mediating immune responses in the lung. Therefore, we suggest that peripheral systemic changes in lipid transport and metabolism can ultimately lead to local lung destruction and the development of emphysema, a novel clinically relevant mechanism for the development of this disease.

Animals	Diet	Surface Area/Unit Volume	Fractional Volume (%)	Mean Linear Intercept, μm	Total cholesterol, mg/dl
$Apoe^{+/+}$ mice (n=6)	Chow diet 10 weeks	**62.4 ± 9.1**	**37.2 ± 3.4**	**32.6 ± 4.7**	**105 ± 25**
$Apoe^{-/-}$ mice (n=6)	Chow diet 10 weeks	**54.7 ± 5.4**	**26 ± 5.1**	**36.8 ± 4 ****	**818 ± 238**
$Apoe^{-/-}$ mice (n=6)	Western-type diet 10 weeks	**48.4 ± 3.2**	**29.8 ± 3.7**	**41.3 ± 2.5 ***	**1118 ± 221**
$LDLr^{-/-}$ mice (n=6)	Chow diet 10 weeks	69.3 ± 10	37.7 ± 2.2	29.3 ± 4.9	358 ± 56
$LDLr^{-/-}$ mice (n=6)	Western-type diet 10 weeks	58.2 ± 3.3	30.9 ± 3	34.3 ± 2.3	1167 ± 327

***p<0.0005** ($Apoe^{+/+}$ chow vs $Apoe^{-/-}$ HF)
****p<0.05** ($Apoe^{-/-}$ chow vs. $Apoe^{-/-}$ HF)

Table 1. Study population
The study population consisted of five groups of mice, all in the C57BL/6J background and subjected to a chow or a Western type, high-fat diet for 10 weeks (n=6 in each group). Morphometric analysis consisting of determination of alveolar surface area per unit volume [S(p=1)], fractional volume of parenchyma tissue per lung [V(p=1)] and mean linear intercept (MLI, microns) was conducted.



Animals	Diet	MLI, µm
Apoe$^{+/+}$	Chow	32.6 ± 4.7
Apoe$^{-/-}$	Chow	36.8 ± 4
Apoe$^{-/-}$	HF	41.3 ± 2.5
LDLr$^{-/-}$	Chow	29.3 ± 4.9
LDLr$^{-/-}$	HF	34.3 ± 2.3

Figure 1. Morphometric analysis of lung tissue of Apoe$^{-/-}$ and LDLr$^{-/-}$ mice.
The mean linear intercept (MLI) was measured in H&E stained lung tissue sections. **A-B.** H&E stained lungs from Apoe$^{+/+}$ and Apoe$^{-/-}$ mice fed a chow diet for 10 weeks. **C.** H&E stained lungs from Apoe$^{-/-}$ mice fed a Western-type diet for 10 weeks. **D-E.** H&E stained lungs from LDLr$^{-/-}$ mice fed a chow and a Western-type diet for 10 weeks. All pictures at 400X magnification. HF = high-fat diet.

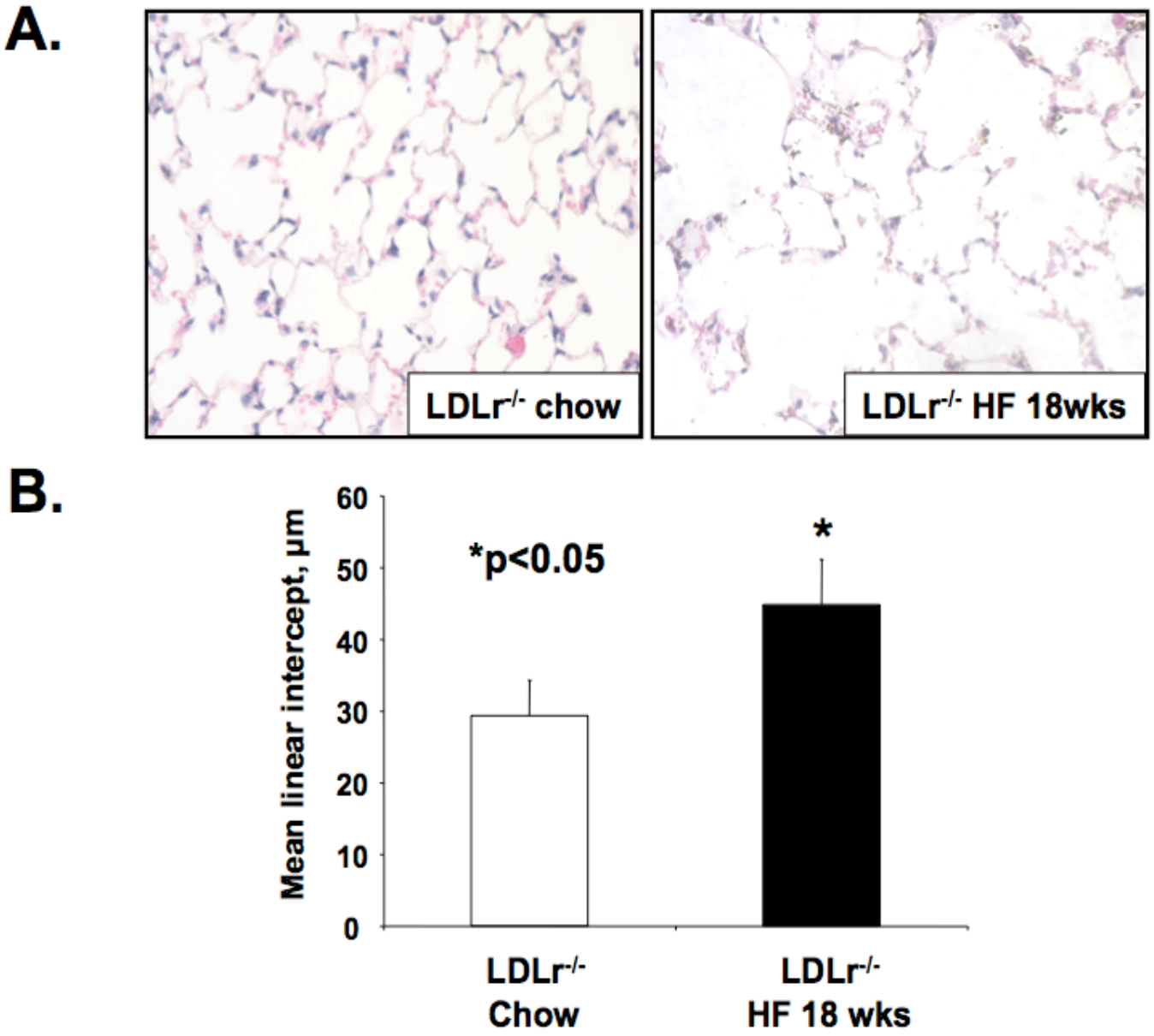


Figure 2. Mean linear intercepts in lungs of LDLr$^{-/-}$ mice after 18 weeks on a chow or on a high-fat diet.
A. H&E stained lungs from LDLr$^{-/-}$ fed a chow diet for 18 weeks and fed a high-fat diet for the same period (400X magnification). **B.** The mean linear intercept (MLI) was measured in H&E stained lung tissue sections from LDLr$^{-/-}$ mice on a chow and on a high-fat diet (n=6 in each group). HF = high-fat diet.

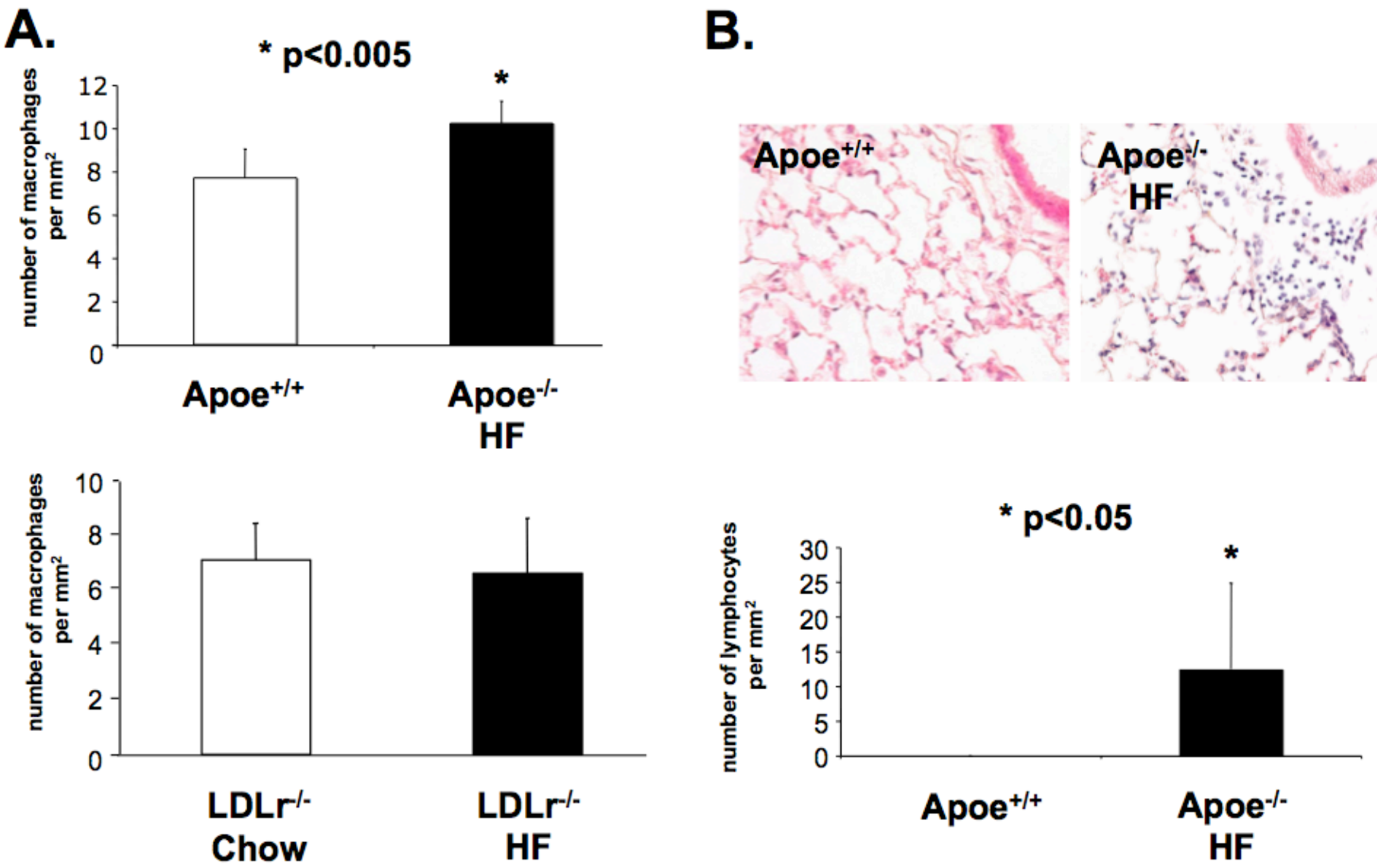


Figure 3. Number of macrophages and lymphocytes in the lungs of Apoe$^{-/-}$ fed a high-fat (HF) diet.
A. The number of macrophages was quantified in the lungs from Apoe$^{-/-}$ and LDLr$^{-/-}$ mice subjected to chow or to a high-fat diet. **B.** Quantification of the number of lymphocytes in Apoe$^{-/-}$ mice. For quantification, six mice in each group were used and, for each mouse, ten pictures (400X) were analyzed. HF = high-fat diet.

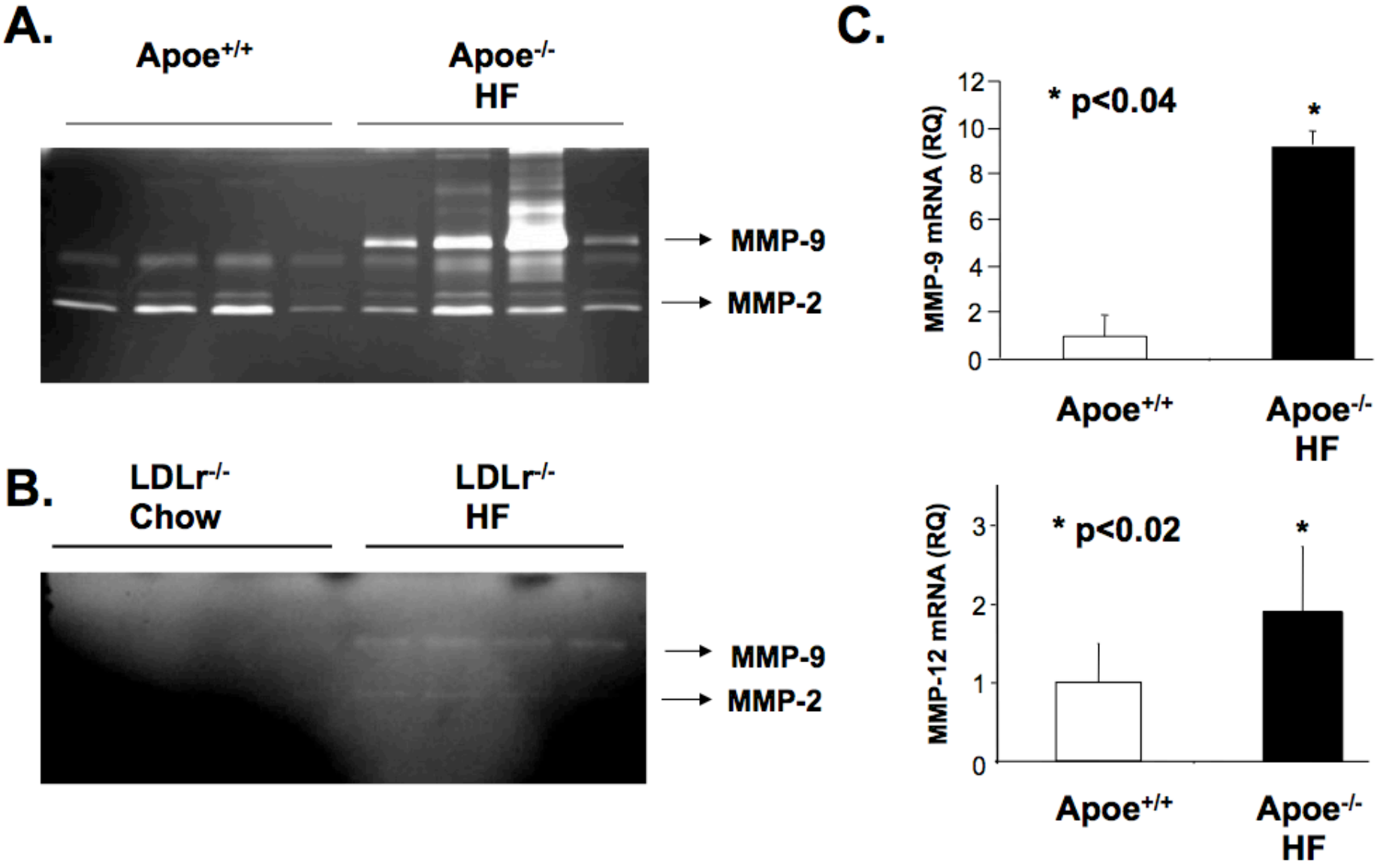


Figure 4. Analysis of MMP-9 and MMP-12 expression in the lungs of Apoe$^{-/-}$ and LDLr$^{-/-}$ mice.
A. MMP-9 activity was analyzed by gelatin zymography in the bronchioalveolar lavage (BAL) fluids of Apoe$^{+/+}$ and Apoe$^{-/-}$ mice subjected to a high-fat diet. **B.** Analysis of MMP-9 activity by gelatin zymography in the BAL fluids of LDLr$^{-/-}$ mice subjected to a chow or to a high-fat diet for 10 weeks. **C.** Real time quantitative PCR for MMP-9 and MMP-12 mRNA expression in lungs of Apoe$^{-/-}$ mice subjected to a high-fat diet for 10 weeks compared to wild-type controls. RQ = Relative quantity, HF = high-fat diet.

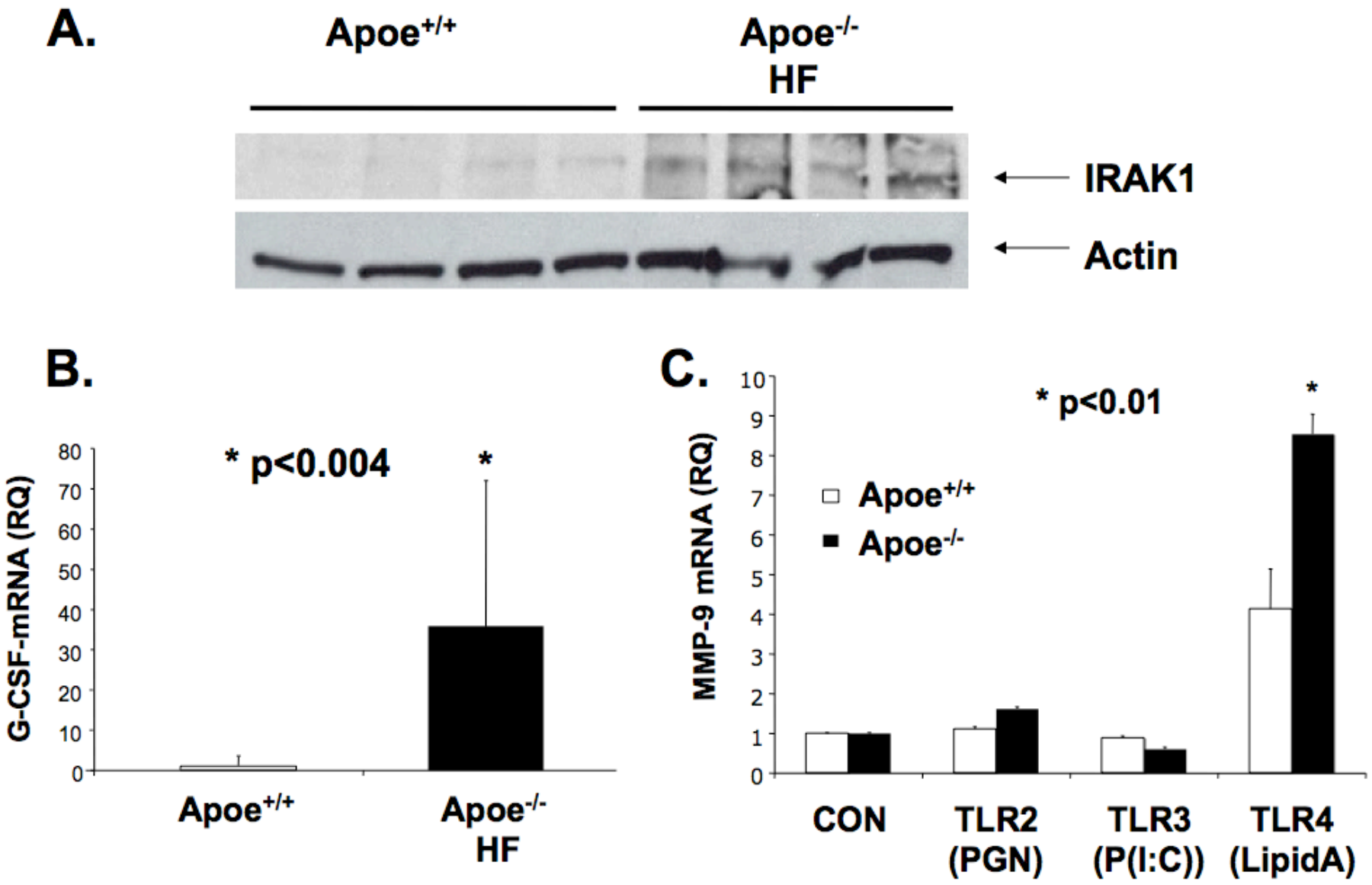


Figure 5. Analysis of TLR signaling in the lungs of Apoe$^{-/-}$ mice subjected to a Western-type diet for 10 weeks.
A-B. Analysis of the expression of downstream regulators of TLR signaling, IRAK-1 (Western blot, panel A) and G-CSF (qRT-PCR, panel B), in the lungs of Apoe$^{-/-}$ mice fed a high-fat diet compared with Apoe$^{+/+}$ mice. **C.** Analysis of MMP-9 mRNA expression in macrophages treated with TLR ligands. Macrophages isolated from Apoe$^{-/-}$ and Apoe$^{+/+}$ mice were cultured in DMEM containing 5% FBS for 24 hours and subsequently incubated with various toll-like receptor (TLR) ligands for 24 hours. TLR2 [peptidoglycan (PGN), 2μg/ml], TLR3 [polyinosine-polycytidylic acid (poly(I:C), 2.5μg/ml], and TLR4 [lipid A, the active component of lipopolysaccharide, 100ng/ml] ligands were used. HF = high-fat diet.

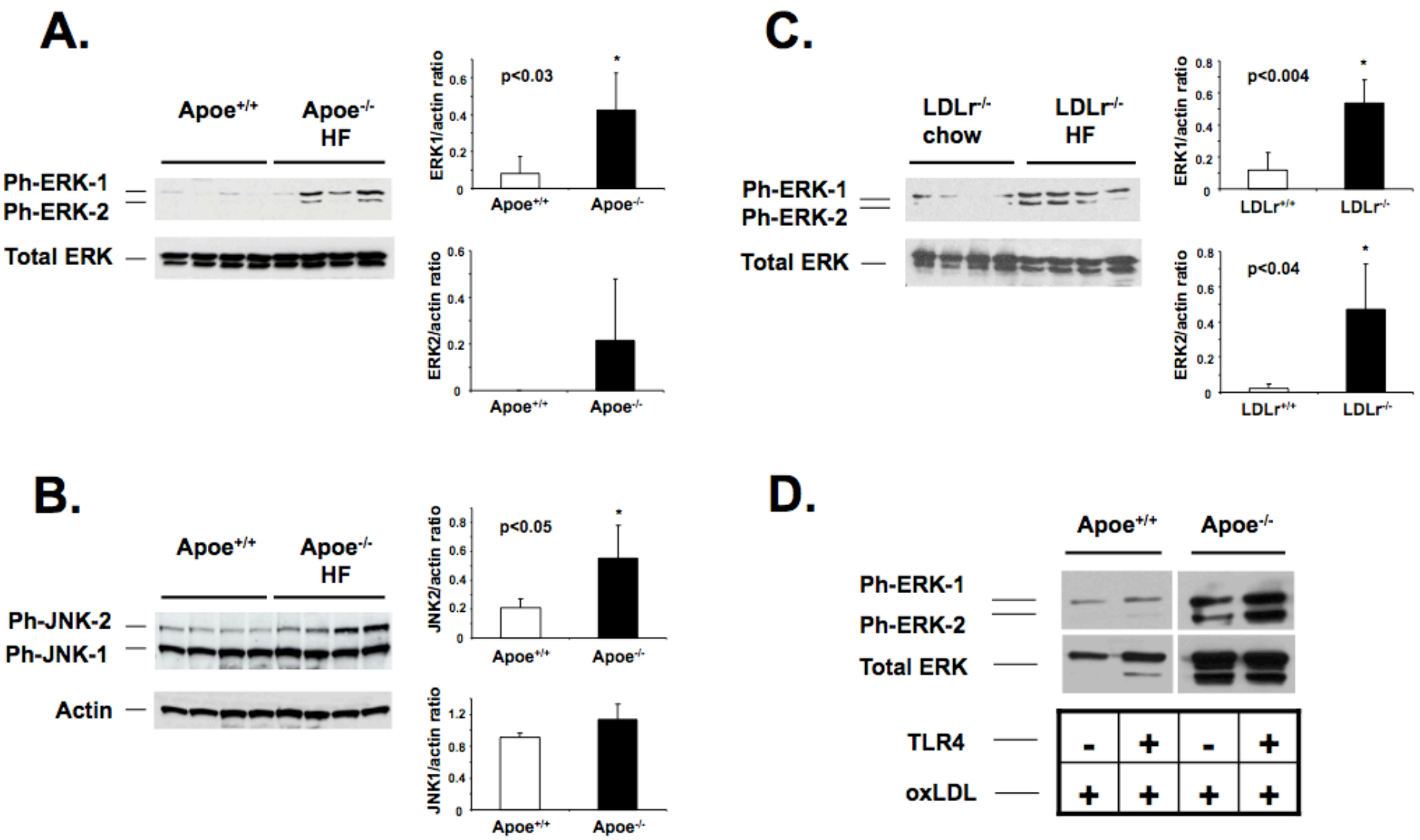


Figure 6. Analysis of ERK and JNK MAP kinases in the lungs of Apoe$^{-/-}$ and LDLr$^{-/-}$ mice.
A-B. Western blot analysis of phospho-ERK (ph-ERK) and phospho-JNK (ph-JNK) in the lungs of Apoe$^{-/-}$ mice after 10 weeks of Western-type diet. Densitometric analysis of the signal was performed to determine ERK/actin and JNK/actin ratio. **C.** Analysis of phospho-ERK in the lungs of LDLr$^{-/-}$ fed a high-fat diet for 10 weeks compared to LDLr$^{-/-}$ fed a chow diet. **D.** Peritoneal macrophages from Apoe$^{-/-}$ and Apoe$^{+/+}$ mice were treated with TLR4 ligand (lipidA, 100ng/ml) and oxidized LDL (100 μg/ml). Activation of ERK was analyzed by Western blotting. HF = high-fat diet.

Chapter 4

The development of emphysema in a rabbit model of atherosclerosis

Abstract

The incidence of chronic obstructive pulmonary disease (COPD) in patients with cardiovascular diseases is undervalued due to a limited knowledge regarding the mechanistic association between COPD and atherosclerosis. Recent studies suggest that the mechanism linking these two diseases can be explained by persistent systemic inflammation. To determine if hypercholesterolemia alters lung inflammation and emphysema formation, we examined the lung phenotype of rabbits at baseline and on a high-fat diet. The rabbits exposed to cigarette smoke were also included in the study population to evaluate the differences between smoke-induced and hypercholesterolemia-induced emphysema. Airspace enlargement developed in the lungs of rabbits maintained on the high-fat diet for 16 weeks. Interestingly, a similar degree of lung destruction was observed in the lungs of rabbits exposed to cigarette smoke. An elevated number of macrophages accompanied by an increase in matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-9 (MMP-9) expression was observed in the lungs of rabbits on a high-fat diet. The augmented activity of neutral sphingomyelinase and increased apoptosis were detected in the lungs of the rabbits on a high-fat diet suggesting that the molecular mechanism leading to the destruction of lung tissue in the hypercholesterolemic rabbits implicates ceramide signaling. Our findings indicate that hypercholesterolemia alone is sufficient to induce pulmonary inflammation and alveolar destruction with subsequent emphysema formation in rabbits.

Introduction

Over twelve million Americans are suffering from emphysema and its complications (Krishnan, 2010). Emphysema is characterized by the destruction of alveolar walls, ultimately leading to the irreversible enlargement of airspaces distal to terminal bronchioles in the lung (Sharafkhaneh, Hanania, & Kim, 2008). The most common comorbidities of emphysema are hypertension, diabetes, heart failure, ischaemic heart disease, cancer, osteoporosis, and anemia (Chatila, Thomashow, Minai, Criner, & Make, 2008; Mannino, Thorn, Swensen, & Holguin, 2008). The coexistence of these diseases clearly affects the mortality rate for emphysema. Determination of the underlying cause of death in patients with multiple diseases is extremely difficult, especially when a common risk factor such as cigarette smoking is involved. Among the numerous environmental and genetic risk factors contributing to the development of emphysema, which includes occupational exposure, air pollution, diet, alcohol consumption, and genetic factors, smoking is the most preventable (Fagerstrom, 2002).

Various animal models have been developed to study the development and progression of emphysema (D'Armiento et al., 1992; R. Foronjy et al., 2008; Hautamaki et al., 1997). Mice exposed to cigarette smoke develop emphysematous changes accompanied by a profound pulmonary inflammation (R. F. Foronjy et al., 2005). Interestingly, recent studies from our laboratory demonstrate the development of a similar pulmonary pathology in *Apoe*$^{-/-}$ mice, a model of atherosclerosis, suggesting that there is a link between atherosclerosis and emphysema. The mechanisms leading to the abnormal lung destruction in *Apoe*$^{-/-}$ mice include increased inflammation, oxidative stress, and an

imbalance between proteases and antiproteases. The excessive activity of matrix metalloproteinases and cathepsins is believed to be responsible for the aberrant disruption of the pulmonary collagen and elastin, resulting in emphysema (R. Foronjy et al., 2008; Shiomi et al., 2003). In accordance with this hypothesis, transgenic mice overexpressing matrix metalloproteinases (MMP)-1 and -9 in the lungs manifested significant emphysematous changes (D'Armiento et al., 1992; R. Foronjy et al., 2008). In contrast, mice deficient in MMP-12 were protected against smoke-induced emphysema (Hautamaki et al., 1997). Additionally, increased cathepsin K has been demonstrated in the guinea pig model of smoke-induced emphysema and in human emphysema (Golovatch et al., 2009). Together, these animal studies suggest that matrix metalloproteinases and cathepsins play a pivotal role in the development of emphysema.

However, the murine model is not the most suited to study the proteases involved in human emphysema, due to differences in enzyme repertoire and physiology compared to humans. In particular, MMP-1, an interstitial collagenase contributing to the destruction of the lung extracellular matrix in human emphysema, is not present in the murine lung (Henriet, Rousseau, & Eeckhout, 1992; Schorpp et al., 1995). Contrary to murine models, the immune response and enzyme repertoire of rabbits are similar to those of humans (Vincenti et al., 1998; Yocum, Lopresti-Morrow, Reeves, & Mitchell, 1999). In addition, the rabbit is an excellent model to study the pathology of atherosclerosis. The rabbit rapidly develops severe hypercholesterolemia, which results in premature atherosclerosis in response to high-cholesterol feeding (Daley et al., 1994). Therefore, the rabbit can be a valuable tool to study the pathology of emphysema in vivo and specifically a link between atherosclerosis and emphysema.

In this study we sought to investigate the impact of a hypercholesterolemic diet on the pulmonary inflammation, proteolytic responses and the development of emphysema in rabbits.

Methods

Experimental design

Twenty female New Zealand White rabbits weighing 1.2-1.3kg were obtained from Covance. The study population was divided into four groups (n=5 in each group) and treated as follows. The first group of rabbits was fed a high-fat atherogenic diet for 16 weeks. The atherogenic diet contained high-fiber rabbit chow (Teklad 2031) with 4.7% hydrogenated coconut oil and 0.15% cholesterol (Research Diets, NJ). The second group of rabbits was exposed to cigarette smoke in a specially designed chamber for 4h/day 5d/week for 16 weeks at a total particulate matter concentration of 100mg/m^3. The third group of rabbits was challenged with both high-fat diet and cigarette smoke for 16 weeks. The fourth group of rabbits served as a control group and therefore was fed chow and exposed to room air for 16 weeks. Animals were housed at Columbia University Medical Center according to animal welfare guidelines. Food and drinking water were provided ad libitum. All animal studies were approved by the Columbia University Institutional Animal Care and Use Committee. Total plasma cholesterol levels were determined by a 4-aminoantipyrine-based enzymatic assay (Wako Bioproducts, VA).

Histology and immunohistochemistry

After 16 weeks of high-fat diet and cigarette smoke, rabbits were sacrificed by carbon dioxide inhalation. The trachea was cannulated with a catheter secured with a silk suture. The lungs were lavaged first with PBS (40ml), to collect bronchoalveolar lavage (BAL) fluid, then infused with 10% formalin for 24 hours. Total cell number from the BAL fluid

of rabbits was counted using a hemocytometer, and a differential analysis was conducted after cytospin preparation and Diff-Quick staining of the cellular pellet prepared in a glass slide. Cells were stained by Diff-Quick, a Romanowsky stain variant, differentiating monocytes, macrophages, neutrophils, and lymphocytes. Tissues were embedded in paraffin and sectioned (6 μm). Sections were stained with hematoxylin and eosin (H&E) for histological analysis. For identification and quantification of macrophages, mouse anti-rabbit monoclonal antibody RAM11 (DAKO) was used. For immunohistochemical analysis, goat polyclonal antibody for MMP-1 (R&D Systems) and goat polyclonal antibody for SP-A (Santa Cruz) were used. Morphometric analysis of the H&E stained lungs of 4 rabbits in each group was performed as previously described (R. F. Foronjy et al., 2006). Morphometric assessment was conducted to determine the average distance between alveolar walls (mean linear intercept). The degree of lung parenchymal destruction was determined by destructive index analysis – a microscopic point count technique. This analysis is performed using a transparent sheet with 50 counting points laid on the microscopic images from the stained lung sections. Ten histological fields (400X magnification) were analyzed from two separate sections from each rabbit (n=4 in each group) to calculate morphometric parameters.

Western Blotting

Freshly dissected lungs of rabbits (10 mg) were homogenized in 1ml of protein lysis buffer (PBS containing Triton X-100 0.1%), and centrifuged (14000g for 10 minutes). Fifty μg of the lung lysates of each group were subjected to Western Blot analysis. Goat

polyclonal antibody against MMP-1 (R&D Systems) was used, following the manufacturer's instructions.

Zymography

Zymography was performed to detect proteases having gelatinolytic activity, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). BAL samples obtained from the rabbits were electrophoresed in gelatin substrate gel and the gelatinases were visualized in a Coomassie-stained gel as clear bands.

Quantitative RT-PCR

Total RNA was extracted from two specimens of lung tissue 0.3cm^3 in size with the use of a RNeasy kit (Qiagen). TaqMan gene expression assays were performed to assess gene-transcript levels with the use of an ABI Prism® 7900HT Sequence Detection System (Applera Corporation, Foster City, CA). Primer and probe set for Cathepsin K (Oc03398668_g1) were purchased from ABI. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed on lung tissue from four animals in each group. To calculate the relative quantity (RQ) of Cathepsin K, we used the $2^{-\Delta\Delta CT}$ method implemented in the software. The data are shown as the fold change in gene expression normalized to the endogenous reference gene GAPDH and relative to the controls.

TUNEL staining

Apoptotic DNA strand breaks were detected in the lung sections of rabbits with the Roche In Situ Fluorescent Cell Death Detection Kit (Roche, Indianapolis, IN) following the manufacturer's protocol. Quantification of apoptotic cells was performed by counting the number of TUNEL-positive cells from 10 images (200X magnification) from each rabbit. The percentage of apoptotic cells was obtained by dividing this count by the total number of nuclei. Total cells were counted by using Hoecht's stain.

Enzymatic Activity Determination

Sphingomyelinase (SM) activity was measured using the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes, Invitrogen detection technologies) following the manufacturer's instructions. In this assay, SM activity was monitored using a fluorogenic probe for hydrogen peroxide (Amplex Red reagent). Amplex Red associates with hydrogen peroxide, which is an ultimate product of SM activity, and generates the highly fluorescent product, resorufin. Hydrogen peroxide and sphingomyelinase were used as positive controls. The sphingomyelinase activity in the lung lysates of rabbits was measured with fluorometer using excitation at 530nm and fluorescence detection at 590nm.

Statistical analysis

Data are shown as mean ±SD. Unpaired two-tailed Student’s t-test analysis was performed to determine statistical significance ($p<0.05$).

Results

Development of severe hypercholesterolemia in rabbits fed high-fat diet

Rabbits maintained on a high-fat atherogenic diet for 16 weeks exhibited a dramatic elevation of their total cholesterol levels and developed severe hypercholesterolemia (1,982 mg/dl vs. 114 mg/dl for the controls) (**Table 1).**

Increased inflammatory cell infiltration in rabbits subjected to a high-fat diet

To evaluate the impact of hypercholesterolemia on pulmonary inflammation in rabbits, bronchoalveolar lavage (BAL) cell counts were performed. There was a considerable elevation in the total cell count of lavage from rabbits fed high-fat diet compared to their controls. A significant increase in the number of macrophages was detected in the lungs of rabbits fed a high-fat diet compared to their controls as determined by differential analysis ($3.2x10^3$ cells/ml vs. $0.2x10^3$ cells/ml for the controls, $p<0.009$) **(Figure 1)**. Macrophages were also stained with anti-RAM11 antibody, which is a specific marker of rabbit macrophages, and positive cells were quantified in tissue sections (114.75 cells/20 fields vs. 16.7 cells/20 fields for the controls, $p<0.004$) **(Figure 2A and 2B)**. Macrophages were the predominant inflammatory cell type in the lungs of rabbits fed high-fat diet. In addition, augmented inflammation and increase in the number of macrophages was observed in the lungs of smoke-exposed rabbits (110.75 cells/20 fields vs. 16.7 cells/20 fields for the controls, $p<0.004$) **(Figure 2)**. There was also an impressive increase in neutrophils in the lung lavage in response to cigarette smoke ($0.9x10^3$ cells/ml vs. $0.07x10^3$ cells/ml for the controls, $p<0.009$) **(Figure 1)**.

Emphysematous changes in the lungs of rabbits due to smoke or to a fed high-fat diet

Emphysema is characterized by an abnormal enlargement of airspaces distal to the terminal bronchiole and accompanied by destruction of alveolar walls. The effect of prolonged hypercholesterolemia on the development of emphysema in rabbits was estimated by measuring the mean linear intercept (MLI). Morphometric analysis demonstrated that rabbits fed high-fat diet for 16 weeks developed statistically significant airspace enlargement compared with their controls (54.5μm vs. 34.5μm for the controls, $p<0.007$) **(Figure 3)**. A destructive index analysis showed a similar pattern when compared to the morphometric assessment of the rabbit lungs (83% vs. 50% for the controls, $p<0.003$) **(Figure 3).** Cigarette smoke induced similar emphysematous changes in the lungs of rabbits (54.25μm vs. 34.5μm for the controls, $p<0.03$) **(Figure 3)**. Importantly, high-fat diet in conjunction with cigarette smoke did not exacerbate emphysema observed in rabbits fed only high-fat diet (46.75μm vs. 54.5μm for the rabbits fed only high-fat diet, $p<0.2$).

Induction of MMP-1 by cigarette smoke and high-fat diet in the lungs of rabbits

Matrix metalloproteinase-1 is expressed in human emphysematous lungs, and our laboratory has identified a critical role for this protease in the disruption of lung collagen during emphysema (D'Armiento et al., 1992; Imai et al., 2001). To determine the modulation of pulmonary MMP-1 due to a high-fat diet, lung lysates from two groups of rabbits were analyzed by Western blotting and ELISA. A marked increase in MMP-1 expression was observed in the lungs of rabbits fed a high-fat diet, compared to the

control group (on a chow diet) **(Figure 4A and 4B)**. Determination of the expression pattern of pulmonary MMP-1 using immunohistochemistry exhibited an increase in signal intensity in the three experimental groups compared to the controls. MMP-1 expression was mainly localized to the alveolar macrophages and type II epithelial cells **(Figure 4C).** We demonstrated through immunohistochemical staining that SP-A-positive cells also express MMP-1 indicating that the expression of MMP-1 was in the type II epithelial cells in the lungs of the rabbits fed a high-fat diet **(Figure 4C)**.

Expression of proteases in the lungs of rabbits

In addition to MMP-1, gelatinase B (MMP-9) has also been implicated in the development of emphysema. In particular we have demonstrated that MMP-9 disrupts the pulmonary elastin when overexpressed in macrophages of transgenic mice (R. Foronjy et al., 2008). In this study, a zymography of the BAL fluids demonstrated low levels of MMP-9 activity in the rabbits fed high-fat diet compared to the control group, where MMP-9 activity was absent **(Figure 4A)**. In contrast, casein zymography analysis showed that MMP-12 levels were similar in the four groups of animals **(Figure 5A)**, suggesting that MMP-12 is not involved in the observed emphysematous changes in the rabbits. Cathepsin K mRNA expression was not altered in the lungs of hypercholesterolemic or smoke-exposed rabbits **(Figure 5B)**.

Alveolar cell apoptosis in the lungs of rabbits

Apoptosis plays an important role in the pathogenesis of emphysema. Several studies demonstrate that there is an increase in the number of apoptotic alveolar epithelial cells in

the lungs of the patients with emphysema (Imai et al., 2005). TUNEL analysis on lung sections revealed a significant increase in TUNEL staining in the lungs of rabbits fed high-fat diet compared to their controls (0.5% vs. 0.09% for the controls, $p<0.002$) **(Figure 6B and 6C)**. Positive and negative- control sections are shown for comparison.

Increased sphingomyelinase activity in the lungs of rabbits

Neutral sphingomyelinase (nSMase) synthesizes ceramide, which is a marker for apoptosis, from membrane sphingomyelin (Petrache et al., 2005). Apoptosis is a key process contributing to the development of emphysema. Since, activation of nSMase stimulates ceramide accumulation and, ultimately, apoptosis, we assessed whether the activity of the neutral sphingomyelinase was modulated in the lungs of rabbits fed a high-fat diet. Interestingly, nSMase was activated in the lungs of rabbits subjected to a high-fat diet for 16 weeks compared to their controls (21.03 mU/mL vs. 14.05 mU/mL for the controls, $p<0.008$) **(Figure 6A)**.

Discussion

The present study establishes that, in rabbits, hypercholesterolemia induces emphysematous airspace enlargement and destruction of alveolar walls. Surprisingly, these morphological changes were comparable to those caused by cigarette smoke exposure alone. High-fat diet-induced and smoke-induced emphysematous changes were both associated with pulmonary inflammation (with an infiltration of macrophages into the alveolar septa), augmented expression of MMP-1 (interstitial collagenase-1) in lung epithelial cells and macrophages, increased alveolar cell apoptosis, and elevated neutral sphingomyelinase activity. These observations suggest that the effects of hypercholesterolemia on lung inflammation and emphysema development are remarkably similar to the effects caused by cigarette smoke exposure.

Numerous studies indicate that an ongoing chronic inflammatory process is a prominent hallmark of emphysema (Hogg et al., 2004; Roth, 2008). In addition, it has been demonstrated that massive pulmonary inflammation aggravates lung injury leading to the formation of emphysema (Mercer et al., 2004; Saetta et al., 2001). Animal models of smoke-induced emphysema have shown that macrophages are the primary cell type contributing to the inflammatory response present in the human disease (Hautamaki et al., 1997; Rangasamy et al., 2004). Our laboratory has also reported that macrophages are the predominant cell type in the BAL fluid and lung parenchyma of smoke-exposed mice (R. F. Foronjy et al., 2005). The present study further emphasizes the importance of macrophages in emphysema, with an increased number of these inflammatory cells in the lungs of rabbits either fed a high-fat diet or exposed to cigarette smoke. A possible

contribution of neutrophils in the development of smoke-induced emphysema has been suggested by a study showing elevated activity of pulmonary myeloperoxidase, a neutrophil marker, in smoke-exposed mice (R. F. Foronjy et al., 2006). In the present study, smoke-exposed rabbits also exhibited a higher number of neutrophils in their lungs, compared to the air-exposed controls, further suggesting a possible interrelationship among neutrophil influx, cigarette smoke and emphysema. However, the number of pulmonary neutrophils was not changed in rabbits fed a high-fat diet, and therefore these cells likely do not play an important role in the formation of emphysema caused by hypercholesterolemia.

Our present study not only demonstrates that hypercholesterolemia induces airspace enlargement in rabbits, but also shows that these pathological changes are comparable with those resulting from chronic exposure to cigarette smoke. Both treatments induced a 50% increase in the mean linear intercept in lungs of rabbits, which is significantly higher than that seen in smoke-exposed guinea pigs and mice (R. F. Foronjy et al., 2005; Golovatch et al., 2009). This finding suggests that rabbits are more sensitive to the development of emphysema compared to other animal models. Higher susceptibility to emphysematous development in rabbits could be explained, in part, by differences in their enzyme repertoires compared to other species. In particular, rabbits, contrary to rodents, possess a homologue for MMP-1, an interstitial collagenase playing an important role in emphysema development in humans (Vincenti et al., 1998; Yocum et al., 1999). Clinical and animal studies have demonstrated that MMP-1 contributes to the disruption of the lung extracellular matrix, leading to the development of emphysema (D'Armiento et al., 1992; Imai et al., 2001; Shiomi et al., 2003). When expressed in the

lungs of transgenic mice, human MMP-1 digests type III collagen fibrils, ultimately causing emphysema. In humans, MMP-1 is detected in emphysematous lungs, mainly in epithelial cells and macrophages, but not in normal lungs, and its expression is induced by cigarette smoke in small airway epithelial cells through the MAP kinase ERK (Imai et al., 2001; Mercer et al., 2004; Segura-Valdez et al., 2000). Here we demonstrate elevated expression of MMP-1 in lung epithelial cells and macrophages of rabbits, either fed with an atherogenic diet or exposed to cigarette smoke, suggesting that this protease participates in the observed emphysematous changes. It is not clear how MMP-1 is induced in the lungs of hypercholesterolemic rabbits, but studies have demonstrated that cholesterol loading triggers MMP-1 expression in macrophages in vitro, suggesting that a similar mechanism may be involved in the alveolar macrophages of rabbits in vivo (Ardans, Economou, Martinson, Zhou, & Wahl, 2002). The contribution of MMP-1 in rabbit emphysema will have to be further investigated, using specific inhibitors of this enzyme in conjunction with a high-fat diet or smoke exposure. In addition to MMP-1, other studies have suggested a role for MMP-9 in the development of emphysema (R. Foronjy et al., 2008; Ohnishi et al., 1998). In the present study however, MMP-9 expression was only slightly elevated in the lungs of rabbits either fed high-fat diet or exposed to cigarette smoke, suggesting that MMP-9 may play a minor role in the observed pathological changes in rabbits.

Alveolar cell apoptosis has been associated with oxidative stress, inflammation, and proteolytic imbalance, and contributes to the initiation and progression of experimental and human emphysema (Kasahara et al., 2000; Tuder, Petrache, Elias, Voelkel, & Henson, 2003; Tuder, Zhen et al., 2003). Studies using mice overexpressing

IFN-γ or deficient in VEGF underscored the importance of apoptosis in the pathogenesis of emphysema (Tang et al., 2004; Zheng et al., 2005). Our laboratory has reported increased alveolar epithelial and endothelial cell apoptosis in the lungs of patients with emphysema (Imai et al., 2005). The present study shows that emphysema in rabbits exposed to a high-fat diet or to smoke is associated with increased apoptosis. This observation suggests that hypercholesterolemia has a comparable effect to cigarette smoke in the induction of alveolar epithelial cells death, a mechanism that likely contributes to the destruction of alveoli. The molecular mechanisms responsible for apoptosis in the lungs of hypercholesterolemic rabbits are not known, but interestingly we detected elevated levels of neutral sphingomyelinase, an enzyme generating ceramide, in the lungs of rabbits either fed a high-fat diet or exposed to cigarette smoke. Patients with emphysema manifest elevated levels of ceramide in their lungs, and it has been shown that inhibition of ceramide synthesis prevents alveolar cell apoptosis, oxidative stress and emphysema in mice (Petrache et al., 2005). Our present study suggests that elevated sphingomyelinase activity secondary to a high-fat diet or to smoke may exacerbate ceramide signaling, leading to an increase in alveolar apoptosis in rabbits. Furthermore, ceramide signaling is a known activator of MMP-1 expression via MAPK pathways, such as ERK1/2, SAPK/JNK, and p38 (Reunanen et al., 1998). Therefore, in addition to apoptosis, ceramide signaling may also be involved in MMP-1 upregulation observed in the emphysematous lungs of rabbits.

Surprisingly, rabbits challenged with both chronic cigarette smoke exposure and a high-fat diet did not develop more severe emphysematous airspace enlargement compared to rabbits exposed to the diet alone or to smoke alone. This observation

suggests that cigarette smoke and a high-fat diet do not act in a synergistic way on the inflammatory response of the lung, and further underlines the complexity of emphysema formation and development.

Our present work emphasizes the importance of hypercholesterolemia and concomitant pulmonary inflammation in the formation of emphysema in rabbits. Studies using animal models and clinical data on human patients have shown that inflammation is a critical event in the development of emphysema. This study provides further evidence for the link between hypercholesterolemia, pulmonary inflammation, and destruction of alveolar walls. In particular, enhanced number of alveolar macrophages, elevated MMP-1, and increased apoptosis may constitute critical and common events in the development of the disease secondary to hypercholesterolemia and cigarette smoke exposure. Further investigation on the relationships between alterations in cholesterol metabolism, lung tissue proteolysis and inflammation is required. An enhanced understanding of the link between cholesterol-induced inflammation and alveolar destruction may provide novel therapeutic approaches for the treatment of emphysema.

Animals	Diet	Exposure	Weight (g)	Cholesterol (mg/dl)
Rabbits (n=5)	Chow	Room air (16 weeks)	4,036 ± 43	114 ± 12
Rabbits (n=5)	Chow	Cigarette smoke (16 weeks)	3,728 ± 127	93 ± 13
Rabbits (n=5)	High-fat diet*	Room air (16 weeks)	4,310 ± 250	1,982 ± 329
Rabbits (n=5)	High-fat diet*	Cigarette smoke (16 weeks)	4,104 ± 182	2,108 ± 149

Table 1. Study population.
The study population was comprised of female New Zealand white rabbits and was divided into four groups (n = 5 in each group). The duration of the treatments was 16 weeks. Body weight (g) and cholesterol levels (mg/dl) were measured in control (fed chow and exposed to room air), fed high-fat diet, exposed to cigarette smoke, and fed high-fat diet and exposed to cigarette smoke rabbits. Data are means ± SD. The high-fat atherogenic diet (*) contained high-fiber rabbit chow with 4.7% hydrogenated coconut oil and 0.15% cholesterol.

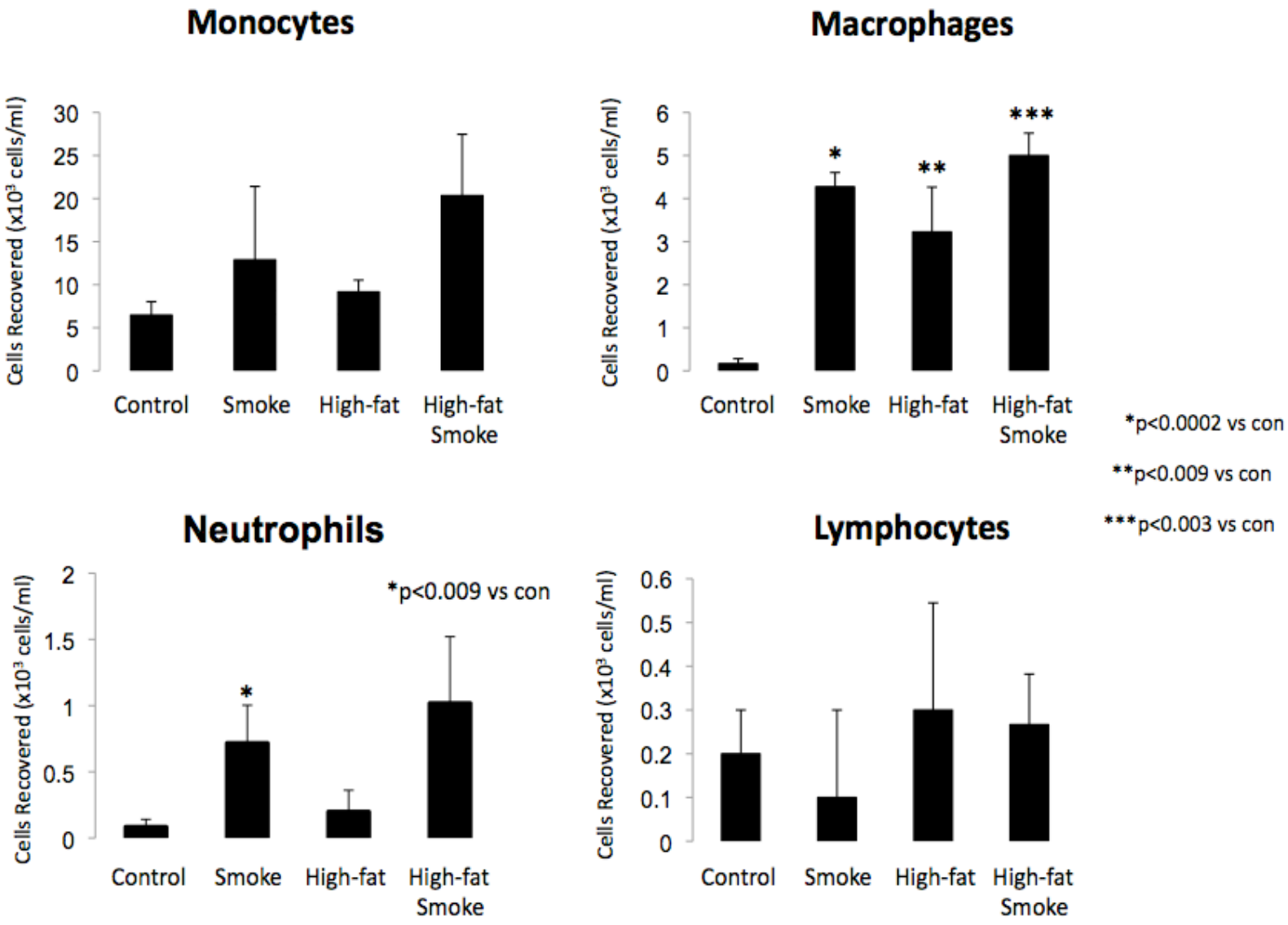


Figure 1. Inflammatory profile of the lung lavage from rabbits.
Total cell number from the bronchoalveolar lavage (BAL) fluid of rabbits was counted using a hemocytometer. BAL fluid was obtained from controls (fed chow and exposed to room air), fed a high-fat diet, exposed to cigarette smoke, and fed a high-fat diet and exposed to cigarette smoke rabbits (n = 4 in each group). Cells were then stained by Diff-Quick, a Romanowsky stain variant, differentiating monocytes, macrophages, neutrophils, and lymphocytes. Cell number is x10^3 cells/mL.

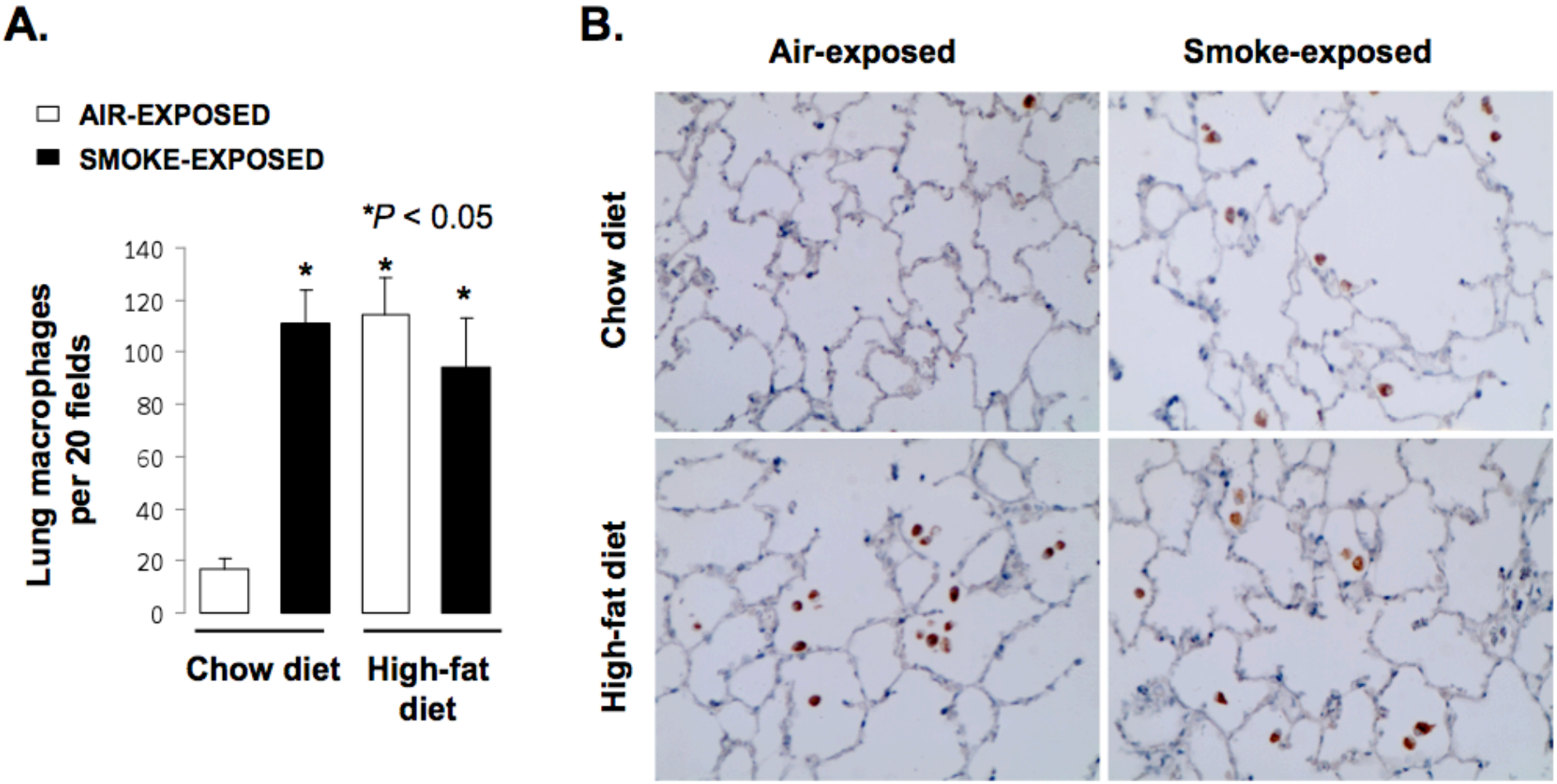


Figure 2. Macrophage numbers in the lung parenchyma of rabbits.
A. Pulmonary macrophages were stained with an anti-RAM11 antibody. The number of macrophages per 20 lung fields was determined for each rabbit (400X magnification). **B.** Immunostaining of lung macrophages using anti-RAM11 antibody. Representative pictures of RAM11-positive macrophages in each group of rabbits are shown above.

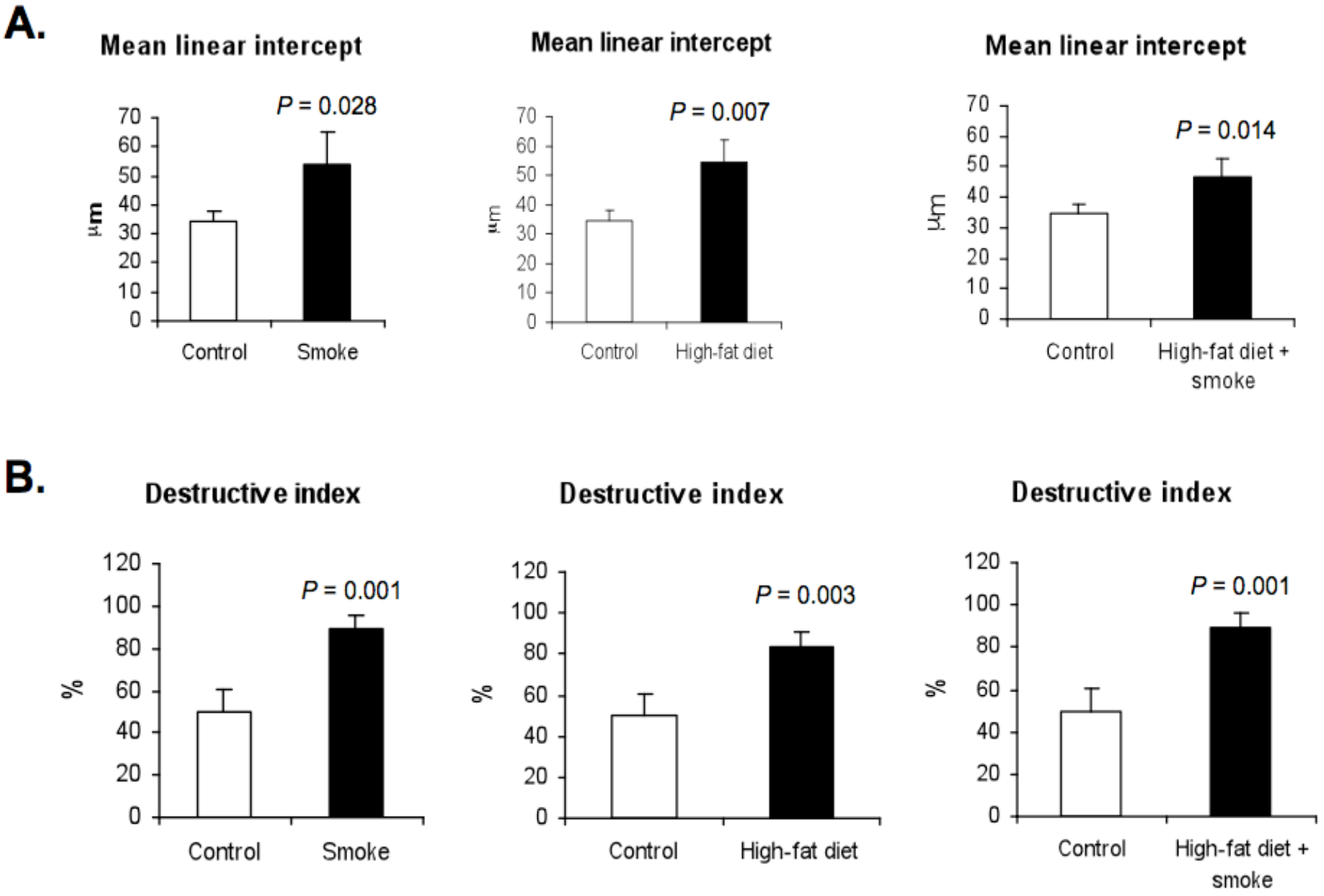


Figure 3. Morphometric analysis of lung tissue of rabbits.
The mean linear intercept (MLI) and destructive index (DI) were measured using H&E stained tissue sections from the four groups of rabbits (n = 4 in each group) (400X magnification) to determine the morphological changes in lungs of rabbits. MLI and DI were assessed as described in Methods section.

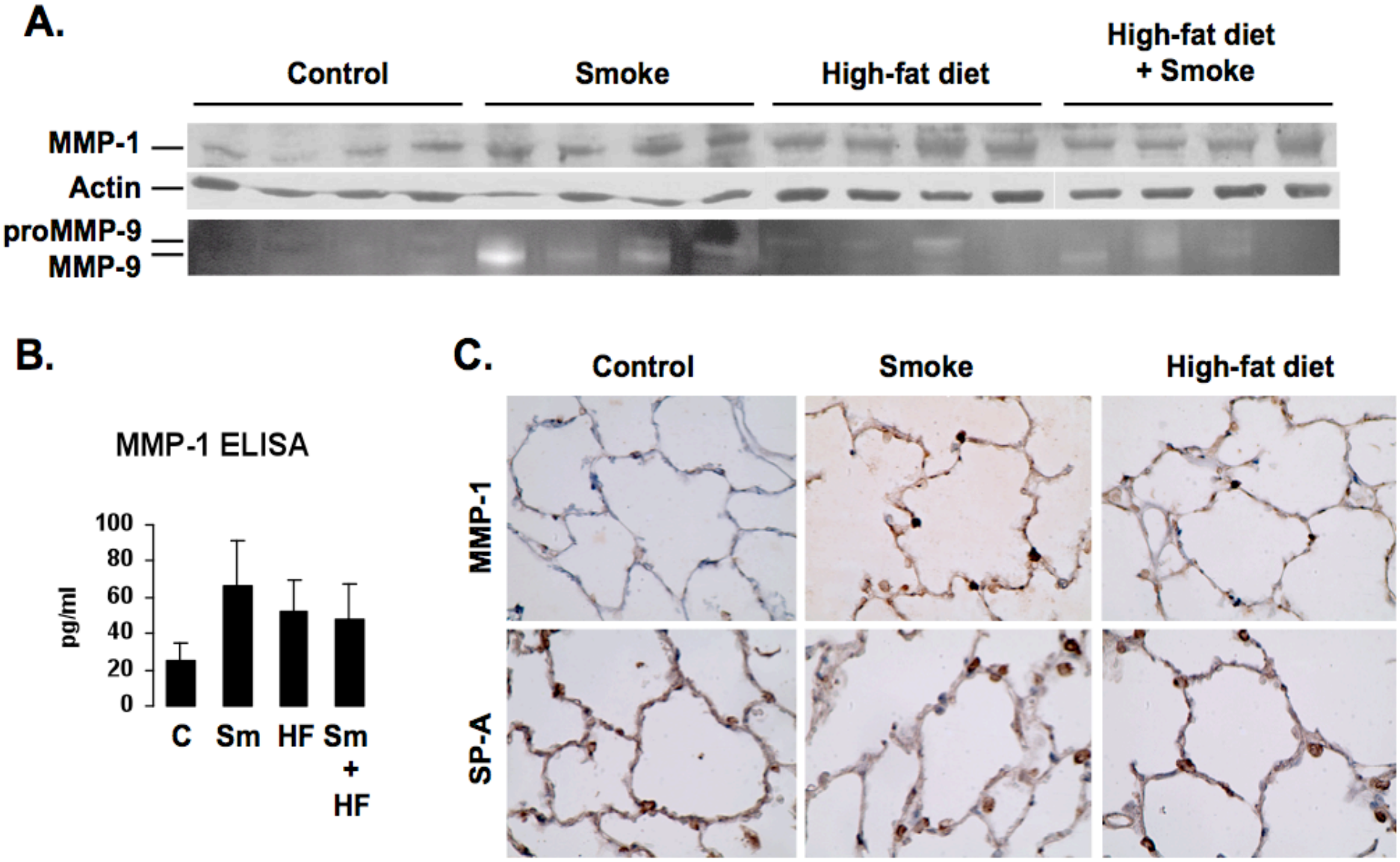


Figure 4. Expression of MMP-1 and MMP-9 in the lungs of rabbits.
A. Lung tissue homogenates from rabbits fed a chow diet and exposed to room air (control), exposed to cigarette smoke (smoke), fed a high-fat diet (high-fat diet), and fed a high-fat diet and exposed to cigarette smoke (high-fat diet + smoke) were analyzed by Western blot for MMP-1 expression. The molecular size of MMP-1 is 52 kDa. MMP-9 activity was detected by zymography in the bronchoalveolar lavage (BAL) fluid of rabbits fed high-fat diet or exposed to cigarette smoke compared to the control group, where MMP-9 activity was not detected. **B.** ELISA of total lung protein extracts demonstrated elevated MMP-1 protein after exposure to a high-fat diet and/or cigarette smoke. **C.** Immunohistochemistry for MMP-1 and surfactant protein A (SP-A), a marker of type II pneumocytes. MMP-1 signal was elevated in the lungs of rabbits exposed to a high-fat diet and/or cigarette smoke and co-localized with alveolar type II cells.

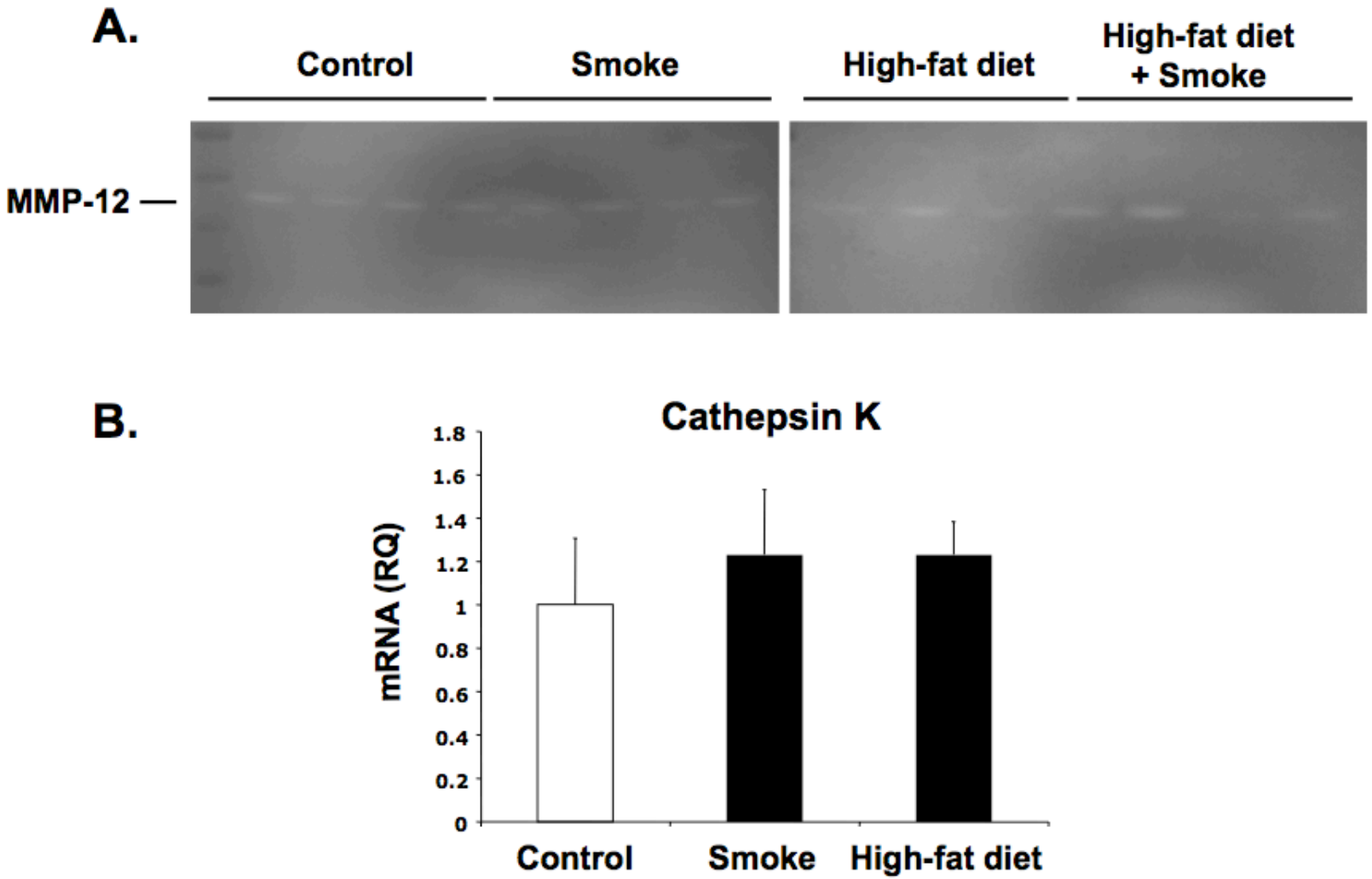


Figure 5. Expression of MMP-12 and Cathepsin K in the lungs of rabbits.
A. MMP-12 activity was detected by casein zymography in the bronchoalveolar lavage (BAL) fluid of rabbits fed a chow and exposed to room air (control), exposed to cigarette smoke (smoke), fed a high-fat diet (high-fat diet), and fed a high-fat diet and exposed to cigarette smoke (high-fat diet + smoke). **B.** Total RNA extracted from the lung tissue of rabbits fed a chow diet and exposed to room air (control), exposed to cigarette smoke (smoke), and fed a high-fat diet (high-fat diet) were analyzed by qRT-PCR for Cathepsin K mRNA expression. Relative quantity (RQ) of Cathepsin K was calculated.

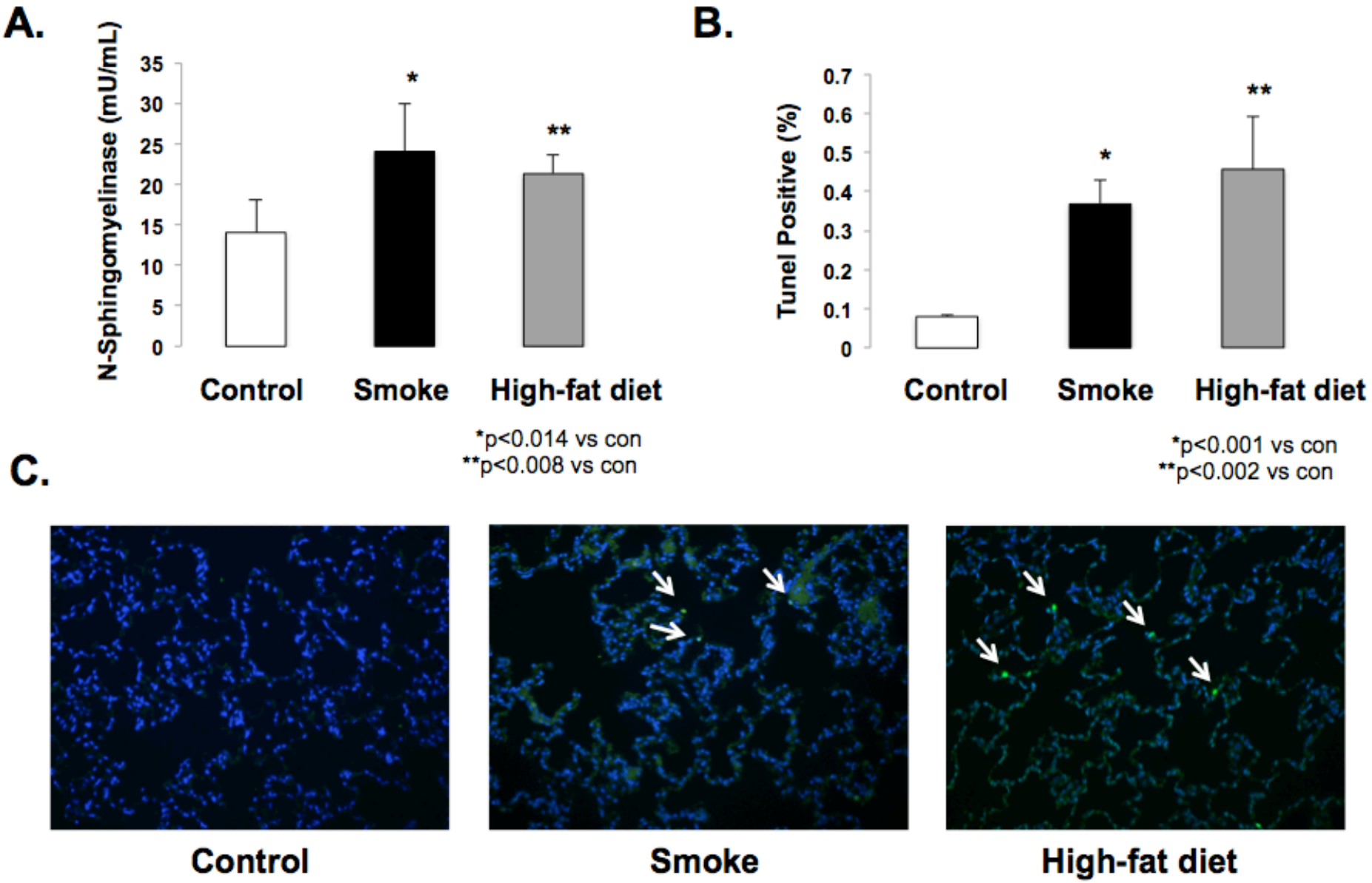


Figure 6. Sphingomyelinase (SMase) activity and alveolar cell apoptosis in the lungs of rabbits.
A. Sphingomyelinase activity assay was performed on the lung lysates from rabbits fed a chow diet and exposed to room air (control), exposed to cigarette smoke (smoke), fed a high-fat diet (high-fat diet). This assay detected the activity of neutral SMase (activity units are mU/mL). **B.** TUNEL staining of lung sections. Quantification of apoptotic cells was performed by counting the number of TUNEL-positive cells from 10 images (200x magnification). **C.** Alveolar cell apoptosis (arrows) detected by TUNEL staining in the lungs of rabbits fed a high-fat diet or exposed to cigarette smoke, compared to their controls (representative pictures).

Chapter 5

Discussion, Summary, and Conclusions

Discussion

It is widely recognized that a greater comprehension of the molecular mechanisms leading to alveolar destruction would aid in the diagnosis and treatment of emphysema. Emphysema is a complex disease influenced by various genetic and environmental factors, and the research work displayed in this thesis contributes to our understanding of this pathology.

The studies presented in this thesis provide novel and interesting insight into the pathogenesis of emphysema and its molecular and cellular determinants. In chapter two, we demonstrate that increased expression of cathepsin K in the lungs of smoke-exposed guinea pigs contributes to the degradation of the pulmonary extracellular matrix, which ultimately results in the development of emphysema. The selection of this animal model for our research is explained by the fact that the development of emphysema in guinea pigs exhibits several similarities to that of the human disease. In particular, guinea pigs develop pulmonary morphological and physiological changes when exposed to the same concentrations of cigarette smoke as humans (Wright & Churg, 2002).

Surprisingly, and despite all these advantages, this animal model has been used extensively in asthma, but not in COPD research. In this second chapter, we not only characterize the guinea pig as a model of smoke-induced emphysema, but we also examine the effect of cigarette smoke exposure on protease expression, MAPK signaling and generation of emphysematous lesions. We identify that smoke-induced emphysema in this model is associated with decreased alveolar type III collagen. This finding is important, since our laboratory has demonstrated that the disruption of this major type of fibrillar collagen leads to emphysema in a transgenic mouse model expressing human

interstitial collagenase (MMP-1) in the lungs (Shiomi et al., 2003). Other studies also reported decreased amounts of collagen associated with emphysematous alveolar destruction in smoke-exposed guinea pigs (Selman et al., 1996; Wright & Churg, 1995). Since only a few enzymes are capable of degrading fibrillar collagens, we first examined the expression of collagenolytic MMPs in the lungs of smoke-exposed guinea pigs. Interestingly, we did not detect changes in the expression of these proteases, leading us to examine another important class of proteases, the cathepsins, which exhibit collagenolytic activity. Together with the MMPs, cathepsins have been shown to be involved in the pathogenesis of emphysema. In particular, mice overexpressing IFN – γ have increased expression of several cathepsins (B, D, H, L and S) in their lungs (Wang et al., 2000). We discovered that the expression and activity of cathepsin K was increased in the lungs of smoke-exposed guinea pigs compared to air-exposed controls. Using human samples, we found that cathepsin K was also significantly elevated in the lungs of patients with emphysema, suggesting for the first time that this protease contributes to the pathological disruption of the lung extracellular matrix in emphysema.

Prior to our discovery, cathepsin K was shown to contribute to another lung disease, idiopathic pulmonary fibrosis (IPF) (Buhling et al., 2004; Srivastava et al., 2008). Since cathepsin K possesses a collagenolytic activity, it is hypothesized that its role in IPF is to attenuate the excessive deposition of pulmonary collagen, which is a prominent characteristic of fibrosis (Srivastava et al., 2008). Our data suggest that, in addition to pulmonary fibrosis, cathepsin K also participates in the remodeling of lung collagen in human emphysema, and provide evidence for a new mechanism of collagenolysis in this pathology.

Research in our laboratory has shown that the ERK, MAP kinase, is activated in human emphysema, inducing the expression of MMP-1, an enzyme critical in pulmonary collagen disruption (Mercer et al., 2004). In our guinea pig model, we also demonstrate increased phosphorylation of pulmonary ERK due to smoke (Golovatch et al., 2009), where it likely contributes to the induction of inflammatory genes. However, the impact of ERK activation on cathepsin K expression remains to be examined. In addition to ERK, other signaling pathways activated by cigarette smoke may contribute to the inflammatory response associated with the destruction of lung parenchyma. In particular, it has been shown that acute exposure to cigarette smoke causes infiltration of neutrophils and activation of NF-kB signaling in the lungs of guinea pigs (Nishikawa et al., 1999).

The study presented in the third chapter focused on the effects of hypercholesterolemia and abnormal cholesterol efflux on the lung extracellular matrix. The lung phenotype of two murine models of atherosclerosis was examined for possible pathological changes, and we found that *Apoe*$^{-/-}$ mice fed a high-fat diet for 10 weeks develop emphysematous airspace enlargement, accompanied by alveolar wall destruction. In contrast, *Ldlr*$^{-/-}$ mice were less susceptible to emphysematous changes compared to *Apoe*$^{-/-}$ mice: pathological disruption of the lung parenchyma was observed in *Ldlr*$^{-/-}$ mice after 18 weeks on a high-fat diet, but no changes were seen after 10 weeks on a the diet. These data suggest that the development of emphysema in *Apoe*$^{-/-}$ mice is not only due to hypercholesterolemia, but also to specific properties of Apolipoprotein E. For instance, absence of ApoE has been shown to impair cholesterol efflux in macrophages (Tall, Costet, & Wang, 2002; Zhu et al., 1998). As a consequence, pulmonary macrophages in *Apoe*$^{-/-}$ mice could release higher levels of inflammatory cytokines and proteases due to

an increased accumulation of cholesterol when fed a high-fat diet, leading to emphysema formation. The critical functions of ApoE in reverse cholesterol transport may therefore explain why the *Ldlr*$^{-/-}$ mice didn't develop the same emphysematous changes observed in the *Apoe*$^{-/-}$ animals.

To investigate the mechanisms leading to emphysema in *Apoe*$^{-/-}$ mice, we analyzed the expression of proteases that are believed to contribute significantly to the disruption of the lung extracellular matrix. We observed that the expression of MMP-9 (gelatinase B) and MMP-12 (macrophage elastase) was elevated in the lungs of *Apoe*$^{-/-}$ mice fed with a high-fat diet. Both MMP-9 and MMP-12 can efficiently digest elastin, which is a major component of the lung extracellular matrix (R. Foronjy et al., 2008; Hautamaki et al., 1997). Overexpression of MMP-9 in transgenic macrophages induces a progressive airspace enlargement in mice, accompanied by a decrease in alveolar elastin (R. Foronjy et al., 2008). Clinical studies identified increased MMP-9 and MMP-8 levels in the BAL fluids from patients with subclinical pulmonary emphysema, further suggesting a role for MMP-9 in this disease (Betsuyaku et al., 1999). An important role for MMP-12 in the pathogenesis of emphysema has also been proposed, since MMP-12-deficient mice are protected against smoke-induced airspace enlargement (Hautamaki et al., 1997). Therefore, in our study, we assert that increased MMP-9 and MMP-12 activity likely contributes to the alveolar destruction observed in *Apoe*$^{-/-}$ mice fed Western-type diet.

TLR4 signaling plays an important role in atherosclerosis (den Dekker, Cheng, Pasterkamp, & Duckers, 2010). Our work suggests that there is also an association between TLR4, atherosclerosis and emphysema. We investigated the transduction

pathways responsible for the emphysematous changes observed in *Apoe*$^{-/-}$ mice fed a high-fat diet and found evidence that activation of TLR4 signaling contributes to the destruction of alveolar septa in these mice. Evidence for TLR4 signaling includes increased IRAK1, activation of downstream targets, ERK and JNK, and high levels of G-CSF expression in the lungs. Moreover, in vitro experiments on *Apoe*$^{-/-}$ macrophages demonstrated that activation of TLR4 signaling by a specific antagonist induced MMP-9 expression. As mentioned above, ERK activation and MMP-9 expression in the lung are two characteristics of emphysematous development. Our data suggest for the first time a link between hypercholesterolemia, TLR4 activation, and the development of emphysema.

In atherosclerosis, activation of the TLR4 signaling has been shown to reduce the expression of major genes involved in cholesterol efflux in macrophages, ultimately causing pathological lipid accumulation in these inflammatory cells (Castrillo et al., 2003). Therefore, in addition to MAP kinases and MMP-9 activation, TLR4 signaling could be important in pulmonary inflammation by inhibiting macrophage cholesterol efflux, although a contribution of this mechanism to lung disease has not been demonstrated yet.

In the fourth chapter, to further understand the genetic and molecular aspects of emphysema, we used rabbits to study the development of this disease due to smoke or to a high-fat diet. Contrary to mice and guinea pigs, rabbits express MMP-1, an enzyme that is believed to be critical in human emphysema (Vincenti et al., 1998; Yocum et al., 1999). Clinical and animal studies have demonstrated that MMP-1 contributes to the disruption of the lung extracellular matrix, leading to emphysema (D'Armiento et al.,

1992; Imai et al., 2001). Since mice do not possess this proteolytic enzyme, genetic modifications are required to investigate the role of MMP-1 in alveolar destruction (D'Armiento et al., 1992). Rabbits naturally express a homologue for human MMP-1 in their lungs and for this reason they potentially constitute a more relevant experimental model to elucidate the mechanisms responsible for the formation of emphysema.

Our studies demonstrate that rabbits maintained on a Western-type diet for 16 weeks developed emphysematous changes similar to those observed in rabbits on a chow diet exposed to cigarette smoke for the same period of time. Surprisingly, rabbits challenged with both cigarette smoke and a high-fat diet developed emphysematous airspace enlargement but its severity was not altered. This observation suggests that cigarette smoke affects the lung extracellular matrix in a complex fashion that involves a broad spectrum of proteolytic degradation, possibly followed by connective tissue protein resynthesis or reorganization. In addition, emphysematous changes in the rabbits were accompanied by an elevation of the number of macrophages, which is a characteristic of the inflammatory response contributing to the development of emphysema. In addition, we detected increased MMP-1 expression in the lungs of rabbits either exposed to cigarette smoke or fed a high-fat diet, suggesting that this collagenase contributes to the observed destruction of the lung parenchyma. This protease has been a major focus of our laboratory for a significant period of time. Initially, it had been shown in our laboratory that mice overexpressing MMP-1 in their lungs develop progressive airspace enlargement due to type III collagen digestion. Later, Imai et al. identified that patients with emphysema have increased MMP-1 expression in their lungs, and Mercer et al. demonstrated that cigarette smoke activates ERK in small airway epithelial cells, leading

to MMP-1 secretion. The induction of MMP-1 in the epithelial cells of the rabbit model allows us to investigate the molecular signaling mechanisms that lead to alterations in the epithelium in vivo. Even though the mechanisms leading to increased expression of MMP-1 in the lungs of rabbits fed a high-fat remain to be elucidated, our study further suggests an important role for hypercholesterolemia and high-fat diet in the pathogenesis of emphysema, leading to a similar inflammation as the one induced by cigarette smoke.

Emphysematous changes in the rabbits were accompanied by an increase in the number of alveolar apoptotic cells. To further understand the mechanisms leading to lung damages and apoptosis in rabbits, we measured pulmonary sphyngomyelinase activity in these animals. Sphyngomyelinase activity generates ceramide, which is involved in pulmonary apoptosis of alveolar epithelial and endothelial cells and in emphysema (Petrache et al., 2005). In the fourth chapter, we demonstrate an increase in sphingomyelinase activity in the lungs of rabbits exposed to cigarette smoke or fed a high-fat diet, compared to the controls. These data suggest that ceramide signaling might be activated in the lungs in response to both cigarette smoke and to high-fat diet. An inducer of oxidative stress, hydrogen peroxide, leads to the activation of ceramides and apoptosis (Goldkorn et al., 1998), and it is therefore possible, that in our rabbit model, ceramide signaling is activated by an oxidative stress resulting from either cigarette smoke or a high-fat diet. In conclusion, our data suggest that elevated sphingomyelinase activity due to cigarette smoke or a high-fat diet may lead to an increase in ceramide signaling, contributing to alveolar apoptosis and emphysematous airspace enlargement in the lungs of rabbits (Medler et al., 2008). However, the precise role of sphingomyelinase and ceramide should be further investigated using specific inhibitors.

In the past, studies involving animals and human subjects have had a major impact on our understanding of disease processes and on the development of drug therapies. Elucidation of biochemical pathways that mitigate inflammation resulted in the development of phosphodiesterase and leukotriene receptor inhibitors (Celik et al., 2005; Price, Chisholm, Ryan, Crockett, & Jones). Phosphodiesterase inhibitors prevent the breakdown of cAMP, thus, decreasing the activity of inflammatory cells (Bourne et al., 1974; Price et al., 2010). Leukotriene receptor antagonists inhibit bronchoconstriction and decrease inflammatory cell infiltration (Celik P, Sakar A, Havlucu Y, 2005). While these are effective treatments for pulmonary inflammation, other therapies targeting proteolytic imbalance, oxidative stress or alveolar apoptosis have not been developed. In addition, the investigation of various genetic factors contributing to COPD susceptibility may help to determine the causes of the disease and explain why not all smokers develop COPD.

The work presented in this thesis deepens our comprehension of the molecular and cellular determinants of emphysema and provides us with valuable knowledge about the factors contributing to the formation of the disease pathology. This research improves our understanding of the adverse effects of cigarette smoking and high-fat diet on the lung inflammation, proteolytic responses and the development of emphysema. However, further investigation about the relationships between alterations in cholesterol metabolism, lung tissue proteolysis and inflammation is required. Specifically, an enhanced understanding of the relationships between cholesterol-induced inflammation and MMP-mediated alveolar destruction is essential.

Summary

The pathology of emphysema was characterized in three different animals: guinea pigs exposed to smoke, *Apoe*$^{-/-}$ mice on a high-fat diet, and rabbits exposed to smoke or fed a high fat-diet. All exhibited airspace enlargement and the destruction of alveolar walls, which are the major characteristics of emphysema. The development of emphysema in these animals is associated with increased inflammation, upregulation of proteases and activation of cellular signaling pathways. Despite all these similarities, each experimental model reveals distinct inflammatory cell profile, enzyme repertoire and signaling cascade involved in the development of emphysema.

Inflammation accompanied by the recruitment of macrophages into the alveolar septa is observed in all our animal models. In addition to macrophages, the lungs of *Apoe*$^{-/-}$ mice exhibited increased number of lymphocytes, which may play a key role in the inflammatory response leading to the development of emphysema in these mice. Infiltration of neutrophils was observed in the lung parenchyma and bronchoalveolar lavage of smoke-exposed rabbits, but not in rabbits fed a high-fat diet. These findings indicate that inflammatory responses resulting from hypercholesterolemia and smoke exposure are not identical, although they result in the same degree of emphysematous changes.

Examination of inflammatory pathways demonstrated activation of MAP kinases in guinea pigs exposed to smoke and in *Apoe*$^{-/-}$ mice fed a high-fat diet. In particular, ERK was elevated in both models, suggesting a key role for this kinase in emphysema development, as observed in the human disease. In *Apoe*$^{-/-}$ mice, TLR4 likely plays a key role in upstream signaling leading to ERK activation and protease expression. However,

the role played by TLR4 in emphysema formation in the other animal models (guinea pigs and rabbits) remains to be elucidated.

Elevated protease activity in the lungs is a common feature of the animal models characterized in this study, suggesting that proteolytic injury contributes to the development of emphysema in these models. In particular, our study reveals that, in every model we used, smoke exposure or a high-fat diet results in an elevation of pulmonary MMP-9, an enzyme participating in elastolysis (Foronjy, 2005). However, the collagenolytic response varies between the species. In guinea pigs, chronic exposure to smoke does not modulate the expression of collagenolytic MMPs, such as MMP-1 and MMP-13. In this model, smoke-induced disruption of the pulmonary collagen is likely due to increased cathepsin K activity, a potent collagenase also expressed in human emphysema. In rabbits, cathepsin K activity was not modulated by smoke or a high-fat diet, but these treatments induced interstitial collagenase (MMP-1) expression. The cellular origin of these collagenolytic enzymes also diverged between guinea pigs and rabbits. Cathepsin K in guinea pigs is expressed in macrophages, while MMP-1, similarly to human emphysema, is induced in type II pneumocytes. These data suggest that the collagenolytic response in the lungs secondary to hypercholesterolemia or smoke exposure is highly dependent on the animal species. MMP-1 is a key player in the pathogenesis of human emphysema, and its induction in type II pneumocytes of rabbits indicate that this model more closely resemble the human disease compared to other animals.

In conclusion, our analysis of mice, guinea pigs, and rabbits as experimental models demonstrates that these animals are all useful to discover new pathways leading to lung dysfunction and emphysema. Since rabbits develop emphysema, express MMP-1 in type II pneumocytes and naturally develop atherosclerosis when fed a high-fat diet, these animals are better suited to explore the link between atherosclerosis, hypercholesterolemia, and emphysema. Rabbits are also an appropriate model to understand the precise mechanisms leading to emphysematous changes secondary to cigarette smoke exposure.

Table 1. Summary of the present study. Animal models of emphysema.

Animal models of emphysema	Guinea Pig	Apoe$^{-/-}$ mice	Rabbits
Treatment	Cigarette smoke	High-fat diet	Cigarette smoke High-fat diet
Treatment duration	12 weeks	10 weeks	16 weeks
Inflammation	Present	Present	Present
Inflammatory cell type	Macrophages	Macrophages, lymphocytes	Macrophages
Proteolytic response	Cathepsin K, MMP-9	MMP-9, MMP-12	MMP-1, MMP-9
Signaling pathway	MAPK signaling	TLR4 signaling	Increased sphingomyelinase

Future studies

The studies presented in this thesis demonstrate that, in animal models, hypercholesterolemia and cigarette smoking can induce inflammation and matrix proteolysis, leading to the development of airspace enlargement. This work not only adds significantly to the field of emphysema research, but also raises numerous interesting questions that will need to be investigated, in order to fully understand the pathogenesis of this disease.

In the third chapter, the development of emphysema was identified in *Apoe*$^{-/-}$ mice fed a high-fat diet for 10 weeks. In *Ldlr*$^{-/-}$ mice, emphysematous changes were observed after 18 weeks on the diet, but not after 10 weeks. Through extensive analysis, TLR4 signaling was established as a principal molecular mechanism leading to the pathological changes in the lung architecture of *Apoe*$^{-/-}$ mice on a high fat diet. However, the status of TLR4 signaling was determined only in *Apoe*$^{+/+}$ on a chow compared to *Apoe*$^{-/-}$ mice on an atherogenic diet. In future experiments, we will further investigate the status of TLR4 activation in lungs of *Apoe*$^{+/+}$, *Apoe*$^{-/-}$, and *Ldlr*$^{-/-}$ mice. These mice will be fed a chow or an atherogenic diet for ten to eighteen weeks, and pulmonary TLR4 signaling will be examined.

To further ascertain the role of macrophage TLR4 signaling in the pathogenesis of emphysema in *Apoe*$^{-/-}$ mice, the use of a conditional knockout model for this receptor will be required. Our laboratory has recently developed a transgenic mouse model that specifically expresses Cre recombinase in tissue macrophages (Morishita et al., manuscript in preparation). These transgenic mice have already been crossed into the Apoe knockout background. A conditional knockout model, where the TLR4 gene is

flanked by Lox sequences, will be generated and crossed into our macrophage-specific Cre expressing, Apoe knockout model. This conditional knockout model will allow us to ascertain the role of macrophage TLR4 in the development of emphysema in *Apoe*$^{-/-}$ mice. In particular, resistance to emphysematous changes in this model would demonstrate that TLR4 plays a central role in lung disease in hypercholesterolemic *Apoe*$^{-/-}$ mice.

In the fourth chapter, the pathogenesis of emphysema was investigated in the lungs of rabbits either fed a high-fat diet or exposed to cigarette smoke. These studies determined that airspace enlargement could potentially result from the upregulation of MMP-1 in the alveolar type II cells, as shown by immunohistochemistry. *In vivo* expression of MMP-1 mRNA in type II pneumocytes can be further assessed by *in situ* hybridization of lung sections. *Ex vivo* studies involving alveolar type II cells and macrophages isolated from rabbit lungs will aid in establishing the molecular mechanisms leading to increased expression of MMP-1 (and other inflammatory molecules) secondary to smoke or to a high-fat diet. Changes in the expression of proteolytic enzymes, in particular collagenases (MMP-1, -13, -8, and -14) and MMP-9, and proinflammatory cytokines that are critical in emphysema, such as IL-13, will be examined in these isolated cells in culture, using quantitative PCR, zymography, Western-blotting, and ELISA. These *in vitro* studies will also focus on transduction pathways, including ceramide, TLR, and MAP kinase signaling. In particular, Western blotting will determine the levels of activated ERK in these cells. Since we have demonstrated that ERK is regulating MMP-1 expression in human lung epithelial cells treated with cigarette smoke extract, the role of this kinase in isolated rabbit lung cells

will be assessed using specific chemical inhibitors, such as PD98059 or siRNA, followed by the analysis of MMP-1 expression. TLR signaling will be also investigated, since TLR4 may be crucial in the development of emphysema in *Apoe*$^{-/-}$ mice. Treatment with various commercially available TLR ligands and inhibitors will allow us to determine the role of these receptors in the modulation of MMP-1 expression in either alveolar type II cells or alveolar macrophages in culture. In addition to TLRs, the potential contribution of ceramide signaling to the development of emphysema in rabbits should also be assessed, since we have shown an elevation of N-sphingomyelinase in the lungs after exposure to smoke or to a high fat diet. Therefore, the levels of ceramides and their derivatives in the lungs of hypercholesterolemic and smoke-exposed rabbits will be evaluated. Individual species ceramides will be measured by liquid chromatography and mass spectrometry. Moreover, rabbit alveolar type II cells and macrophages in culture will be treated with ceramide and the consequences of this treatment on MMP-1, proteases, and cytokines expression will be assayed using real-time PCR and ELISA. Studies involving the inhibition of ceramide pathway, using siRNA, GW4869 (an inhibitor of N-sphingomyelinase), and fumonisin B1 (a ceramide synthase inhibitor), will further clarify the role of ceramide signaling in the development of emphysema secondary to smoke or to a high-cholesterol diet. Another useful model to study the effects of hypercholesterolemia on lung disease is the Watanabe heritable hyperlipidemic (WHHL) rabbit. These rabbits have deficient LDL receptor activity due to a deletion in the LDL-binding domain (Yamamoto et al., 1986), leading to hypercholesterolemia (average plasma cholesterol levels of 700 to 1200 mg/dl) and to the development of complicated lesions of atherosclerosis (Shiomi and Ito, 2009). WHLL rabbits have been

used as a model to study human familial hypercholesterolemia and have also helped in the development of drugs aimed at lowering cholesterol levels and preventing atherosclerosis (Shiomi and Ito, 2009). Presence of emphysematous changes and pulmonary inflammation in these rabbits would further demonstrate the existence of a link between hypercholesterolemia and emphysema formation. Moreover, the WHLL rabbits could be useful to determine the effects of cholesterol-lowering drugs, such as statins, on the development of emphysema.

Since we have demonstrated that hypercholesterolemic and smoke-exposed rabbits exhibit increased expression of MMP-1 in their lungs, the studies demonstrating the direct link between this enzyme and emphysema formation in a rabbit model are necessary. The contribution of MMP-1 to rabbit emphysema can be further investigated, using specific inhibitors of MMP-1 activity in conjunction with high-fat diet or smoke exposure. In addition, studies blocking upstream regulators of MMP-1 gene expression, such as TLR4 or ceramide, can represent an alternative means of treating protease-driven emphysema. The absence of emphysematous changes in the rabbits fed high-fat diet or exposed to cigarette smoke would further clarify the role of MMP-1 in the development of emphysema in rabbits.

Finally, further epidemiological studies are required to better understand the effect of hypercholesterolemia on pulmonary inflammation and lung function in humans. These studies will determine the key molecular players associated with the correlation between obesity, cholesterol levels, and a decline in lung function in non-smokers demonstrated in our study. A direct clinical correlation between cigarette smoke induced lipids and subsequent innate immunity induced MMP expression would be of great

interest in the human disease. Clinical studies can also evaluate ceramide signaling in the lungs of patients with emphysema, using immunohistochemical analysis and activity assay.

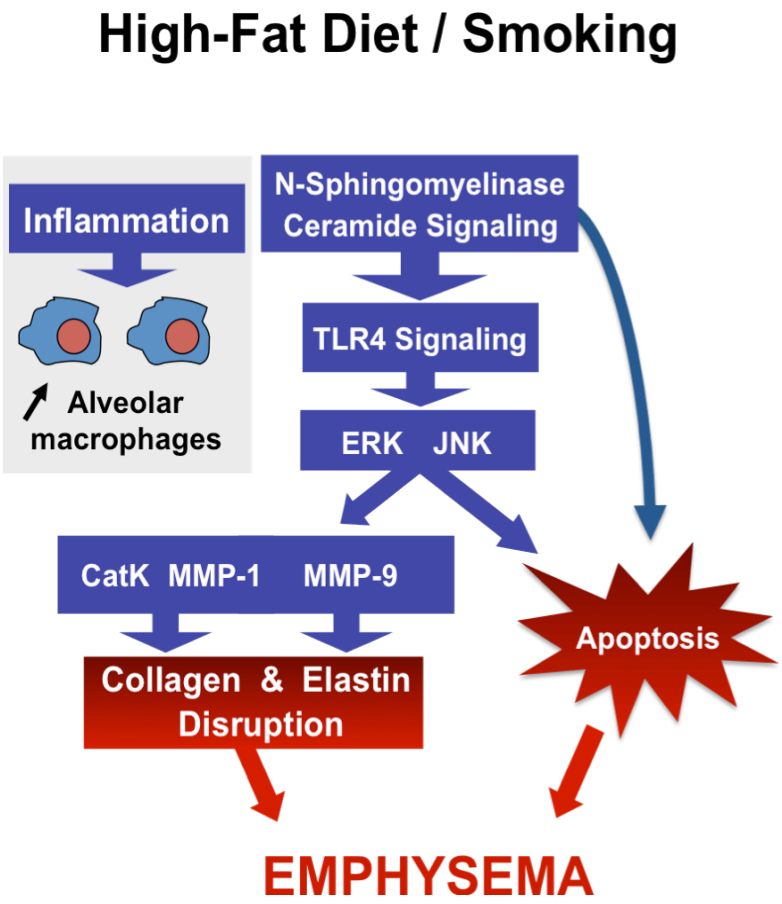


Figure 1. Potential mechanism for the development of emphysema secondary to a high-fat diet or cigarette smoke. Hypercholesterolemia (due to a high-fat diet) and/or exposure to cigarette smoke induce an inflammatory response in the lung, characterized by an accumulation of alveolar macrophages. At the molecular level, hypercholesterolemia and/or exposure to cigarette smoke cause the activation of sphingomyelinase, leading to ceramide signaling, followed by a cascade involving TLR4, and ERK and JNK MAP kinases. This signaling cascade results in the induction of apoptosis and in the release of collagenolytic cathepsin K and MMP-1 and elastolytic MMP-9. Digestion of pulmonary elastin and collagen, together with increased apoptosis, will ultimately result in the development of emphysema.

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